THE ECOLOGY AND PHYSIOLOGY OF COLD-HARDINESS OF OVERWINTERING MONARCH BUTTERFLIES IN MEXICO

BY

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Almost the entire eastern North American population of the monarch butterfly migrates each autumn to overwinter at high-altitude sites in central Mexico. Although these sites are usually cool and stable through the winter, occasional episodes of freezing weather occur. The fact that most of the overwintering butterflies survive these periods of cold suggests that the monarchs have evolved some mechanism of freeze-protection.

Experiments determined that migrating and overwintering monarchs supercool to lower temperatures than freshly eclosed or summer reproductive monarchs or butterflies from two other closely related Danaine species. Furthermore, a daily change in supercooling ability was observed in overwintering monarch butterflies—the freeze resistance is more pronounced at night. Although circadian change in cold-hardiness has been found in an Andean plant, this is the first discovery in insects. Monarch supercooling points (SCPs) were altered experimentally by treatment with methoprene, an insect juvenile hormone (JH) analogue, which is evidence linking the cold-hardiness with other physiological changes of diapause under hormonal control. The pattern of daily change in cold-hardiness found in these butterflies is probably an adaptive response to the tropical high-altitude environment of the Mexican overwintering sites.

However, two ecological factors strongly influence this capacity to resist freezing: wetting and exposure to the night sky. (1) If monarchs are wet, supercooling ability is thwarted and the freezing of individual butterflies is probable at higher sub-zero temperatures (Mean = -4.18°C) compared to freshly collected butterflies with no surface water (Mean = -7.7°C). Measurements of rainfall within a large monarch colony confirmed that the canopy cover aids in protection from wetting. (2) If a monarch is exposed to the night sky, radiational cooling results in a body temperature below both the temperature of ambient air and other butterflies less exposed. Nighttime body temperatures of monarchs under dense cover were approximately the same as ambient air temperature, but body temperatures of monarchs more exposed fell below ambient (as much as 4 degrees) in direct proportion to degree of exposure. Both wetting and exposure are related to the amount of forest canopy overhead and to the position of monarchs within the clusters.

Monarchs from different parts of the migratory cycle were analyzed for glycerol and other carbohydrates. Both glycerol and trehalose concentrations were significantly higher in overwintering butterflies than in monarchs collected from the summer. The daily pattern of SCP change observed in Mexico can be explained by changes in trehalose (but not glycerol) concentrations. Both glycerol and trehalose probably act as cryoprotectants in overwintering monarch butterflies in Mexico.

CHAPTER 1
INTRODUCTION

Cold Selection

The freezing point of water is a temperature of great significance for living organisms. With rare exception, intracellular freezing is lethal to cells (Somme, 1982). Intricate biological organization is usually disrupted irreversibly by the crystalline order of ice. In addition to mechanical damage, osmotic stress and lipid phase-transition effects during freezing tend to overwhelm homeostatic processes in cell membranes (Fahy, 1986; Quinn, 1985). Throughout evolutionary time, ambient temperatures below 0°C have acted as a pervasive selective force on many species, excluding from future generations those genotypes not coding for some behavior of cold-avoidance or physiology of cold-hardiness.

Frigid temperatures can occur over much of the planet. Generally, it is colder toward the poles and with increasing altitude, with annual and daily cycles of change superimposed on this geographic pattern. Annual changes in the global distribution of solar energy, caused by the tilt of the earth's axis of rotation 23.5° from the plane of its rotation about the sun, are responsible for the major seasonal changes in temperature, precipitation, and atmospheric and oceanic currents (Pianka, 1983). The amplitude and mean of seasonal temperature cycles have shifted throughout geologic history, perhaps due to changes in the atmosphere (Graedel and Crutzen, 1989), or variations in the orbit or rotational axis of the earth (Covey, 1984), or changes in sunspot activity (Schove, 1987), or other extraterrestrial influences (Rampino and Stothers, 1986). Recent evidence suggests that the eight glacial cycles within the past million years were the result of periodic reorganizations of the ocean-atmosphere system caused by combinations of astronomical factors (Broecker and Denton, 1990). For thousands of years during these recurrent ice ages, freezing temperatures were prevalent over major parts of the globe, and even during relatively warm periods (such as the present), temperatures seasonally fell below zero in many temperate, boreal, and alpine habitats.

Of all animals, terrestrial poikilotherms are most at risk of having their cells freeze, and many species have evolved physiological mechanisms of cold-hardiness. Aquatic creatures are less threatened at most latitudes — one consequence of the polar asymmetry of the water molecule is a high specific heat and a considerable thermal inertia which attenuates temperature extremes. However, toward the Arctic or Antarctic, aquatic poikilotherms do have to contend with severe cold, especially in intertidal zones, and many of these species are very cold-hardy (Block, 1982a). Homeotherms, unless they run short of metabolic fuel or experience extremes of exposure, keep their body temperatures well above freezing (although arctic ground squirrels have recently been discovered to hibernate at body temperatures below 0°C) (Barnes, 1989). The most successful terrestrial poikilotherms, insects, have evolved an assortment of adaptations to periodic winter, including diapause-mediated dormancy, migration, polyphenism, and various cold-hardiness mechanisms (Tauber et al., 1986).

This dissertation is concerned with the extent and nature of cold-hardiness in a long-distance migratory insect, the monarch butterfly (Danaus plexippus, L.). In the introductory chapter I will first consider aspects of monarch life history to clarify the reasons for the study. Then I will review what is known about the extent and biochemical mechanisms of insect cold-hardiness. Finally, I will describe the hypotheses and predictions that were tested experimentally.

The Monarch Butterfly

Among insects, the monarch butterfly is one of the most striking and well studied examples involving seasonal migration to an overwintering site (Tauber et al., 1986). Each spring monarchs disperse from Mexico and colonize the gulf states of the U.S. (Cockrell et al., In Press). Through the summer, they undergo three to five generations of population expansion on milkweeds (Asclepias sp.), spreading north and eastward well into southern Canada (Brower, 1985). The southernmost U.S. becomes too hot for larval viability by midsummer (Malcolm et al., 1987), but vast areas of milkweed resource across the central and northeastern U.S. and Canada are accessed by this highly mobile insect. In the western U.S., a smaller population of monarchs migrates from overwintering sites along the California coast to summer milkweed ranges west of the Rocky Mountains (Nagano et al., In Press; Tuskes and Brower, 1978).

Each autumn, American monarchs east of the Rocky Mountains abandon their northern breeding ranges and migrate southwestward. Some spend the winter along the western coast of Florida (Baker, 1978; Brower, 1985), but most of the butterflies gather into dense overwintering aggregations high in the transvolcanic mountains of central Mexico (Brower, 1977; Brower, 1985; Calvert and Brower, 1986; Urquhart and Urquhart, 1976; Urquhart and Urquhart, 1977). Within limited boreal forests of oyamel fir (Abies religiosa H.B.K.) only found on the higher mountains, monarch colonies form each autumn at a few sites on southwestern slopes between altitudes of 2,300 and 3,100 m (Calvert and Brower, 1986). At these elevations, ambient temperatures are sufficiently cool and stable throughout the winter to allow most of the butterflies to conserve lipid reserves (Masters et al., 1988) and to remain in a state of reproductive inactivity until just before the spring emigration (Barker and Herman, 1976; Herman, 1981; Herman, 1985). In general, the balance of moisture, insolation and temperature, which allows the butterflies to fly occasionally to drink water as well as to reposition themselves within the clusters, seems ideally suited for monarch survival and for maintenance of the dense colonies (Alonso et al., In Press; Calvert and Brower, 1986; Masters et al., 1988).

However, extreme temperature fluctuations can occur at these altitudes, and despite the ameliorating influence of the forest canopy (Calvert and Brower, 1981), overwintering monarchs are sometimes exposed to freezing cold. During January, the coldest month, sub-zero temperatures are frequent at night in open areas, especially close to the ground (Alonso et al., In Press; Calvert and Cohen, 1983; Calvert et al., 1982; Calvert et al., 1983). Figure 1-1 shows the relationship between altitude and extreme low temperatures (recorded at nine weather stations in the Mexican transvolcanic mountains) (Anon., 1982). The most severe episodes of freezing weather occur during or immediately after winter storms, which have been observed several times since the discovery of the Mexican overwintering sites in 1974 (Calvert and Brower, 1986; Calvert et al., 1989; Urquhart and Urquhart, 1976). The most intense of these storms occurred in January 1981 at the monarch site on the Sierra Chincua in Michoacan, Mexico. Three days of moderate cold, daily rain and hail were followed by a nine-day period of sub-zero temperatures when the monarchs were assaulted by alternating snow, hail, freezing rain and high winds. Cumulative snowfall was 43 cm. On two nights the sky cleared completely, and the average air temperature within the colony (i.e., not exposed to the night sky) fell to -4.1°C, with readings as low as -5°C at several locations (Calvert and Brower, 1986; Calvert and Cohen, 1983; Calvert et al., 1989). Although the authors estimated that 2.5 million monarchs were killed during the storm, the majority of the colony (approximately 80%) survived. These observations indicate that cold temperatures at the overwintering sites can certainly be dangerous for the monarchs. However, the relatively low mortality during the storm of 1981 poses questions as to (1) the reasons for differential mortality and (2) whether monarchs have some physiological mechanism of freeze resistance.

The Oyamel fir forests of Mexico are relicts of boreal-like forests that were much more extensive during the glacial and interglacial periods of the Pleistocene. They are now

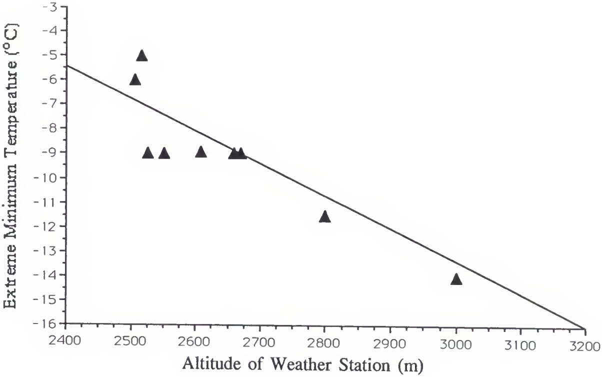

Figure 1-1. Relationship between altitude and extreme minimum temperature recorded at nine weather stations in the Transvolcanic Range near the monarch butterfly overwintering sites over 10 - 26 years (Linear regression, $y = -0.01x + 29.77$, $r^2 = 0.81$, $P = 0.001$). (Anon., 1982)

'island' ecosystems, limited to high mountain peaks at elevations between 2400m and 3600 m (Rzedowski, 1978) — a patchy distribution that makes these forests more vulnerable to deforestation pressures than any other forest type in Mexico (Snook, In Press). However, logging is an important industry in these areas. There is evidence that thinning the forest results in increased butterfly mortality from freezing (Calvert and Brower, 1981; Calvert et al., 1982), and probably more bird predation (Brower and Calvert, 1985a). Therefore, even high quality forestry practice might change the overwintering habitat to the detriment of the monarchs (Brower, 1985). Because the Oyamel forests are in the process of an almost inexorable degradation by human pressure (as are so many other natural habitats today (Wilson, 1989), information as to the exact limits of monarch cold endurance might be helpful, both in predicting the outcome of present trends, and in planning management strategies for future conservation of the monarch butterfly.

Cold and Insect Survival

Many insects survive intense cold during winter. Not long after the invention of the thermometer, Reaumur (1734) noticed that some insect larvae were able to withstand -9°C without perishing. Since then, investigators have discovered cold-hardiness in numerous insect species. Typically, this involves a seasonal increase in the capacity to tolerate or resist freezing (Baust, 1981; Somme, 1982). Some insect eggs and larvae can endure drastically low temperatures in winter, e.g., survival at -51°C for eggs of Norwegian tortricids, Zeiraphera diniana, (Bakke, 1969), -42°C for aphid eggs (Pterocomma smithis) in northern Alberta (Somme, 1964), and -61°C for larvae of a species of gall forming midge (Rhabdophaga sp.) in the North Western Territories. However, these are extremes of cold endurance; most of the eggs and larvae studied can only withstand temperatures down to about -35°C.

In general, the pupae that have been studied are not quite as freeze resistant. Only pupae of a few species can live through temperatures below -30°C, including the noctuid Apatele psi from Estonia (Hansen, 1971), a gall-forming dipteran from Alberta (Somme, 1964), and pupae of the solitary bee Megachile relativa and one of its parasites (Krunic and Radovic, 1974).

Cold tolerance has been studied in several adult insects, including some species of beetles overwintering in the soil, leaf litter or rotten wood (Hoshikiwa et al., 1988; Merivee, 1971; Somme and Zachariassen, 1981), a few species of adult Hemipterans (Decker and Maddox, 1967; Eguagie, 1974), Collembolans (Block, 1982b; Block and Zettel, 1980; Somme, 1979; Somme and Block, 1982; Somme and Conradi-Larsen, 1977), Dipterans (Hudson, 1978), Hymenopterans (Diffie and Sheppard, 1989; Maavara, 1971; Somme, 1964), and Lepidopterans (Frankos and Platt, 1976; Lozina-Lozinskii, 1974; Miller, 1982; Pullin and Bale, 1989). In general, these adult insects have less cold tolerance than the above examples from other life stages, possibly because they can behaviorally avoid cold extremes by choosing protected hibernacula. The most cold resistant adult insects are those overwintering in more exposed habitats, such as cerambycid beetles under bark in Norway and Estonia, or Collembolans in lichens on exposed rocks in the Austrian Alps. These extreme examples can survive temperatures below -30°C, but most cold-hardy adult insects show mean lethal limits from -5°C to -30°C. MacPhee (1964) and Somme (1982) suggested that the cold-hardiness of many overwintering insects is suited only to their average winter conditions and protection offered by their microhabitat, with moderate to high mortality occuring in severe years.

Insect Cold-hardiness Mechanisms

In the literature on insect cold-hardiness, a dichotomy is drawn between freezing-tolerant and freezing-susceptible species (Salt, 1961). Only a few insect species can tolerate internal ice formation (freezing-tolerance), either by directing ice away from intracellular tissues with extracellular nucleating agents, or by somehow enduring the presence of ice within their cells (Miller, 1982; Miller, 1969; Zachariassen and Hammel, 1976). Most insect cold-hardiness, however, is found in freezing-susceptible species (for which freezing is lethal) and works by preventing the formation of internal ice. Resistance to freezing is due to increased concentrations of antifreeze biochemicals that can function in three ways: (1) by lowering the freezing point of body fluids, (2) by lowering the supercooling point of body fluids, or (3) by inhibiting the growth of ice crystals within the body, producing an effect called "thermal hysteresis". For clarity, I will briefly define these concepts.

The freezing point of a solution is the highest temperature at which an ice crystal will grow during gradual cooling and the melting point is the temperature at which an ice crystal begins to shrink during gradual warming. These two temperatures are usually the same. Freezing point depression is directly related to the number (rather than the kind) of solute molecules dissolved. Each mole/liter of solute lowers the freezing point of an aqueous solution by 1.86°C. Because such large concentrations of solute are required to provide relatively small freezing point depressions, this mechanism is biologically impractical (although it is the principle of automobile antifreeze). Nevertheless, some insects are able to survive extreme boreal winters by achieving internal concentrations of cryoprotectants that are surprisingly high, up to 30% of fresh weight (Somme, 1982).

The supercooling point (SCP) of a solution is the highest temperature at which spontaneous freezing occurs during gradual cooling when there is no seed crystal present (Salt, 1961; Zachariassen, 1980). In the absence of ice crystals or nucleating agents, pure water and aqueous solutions will commonly supercool and persist in the liquid phase at a temperature below the freezing point. This state is unstable; and as the temperature decreases, there is an increasing probability that some water molecules will organize as a microscopic seed crystal, after which freezing rapidly progresses by crystal growth. The sudden transition from supercooled liquid to frozen solid is the SCP, which can be conveniently measured as the lowest temperature reached before the occurrence of a sharp increase in the temperature of the solution as the latent heat of fusion of water is released. Small molecular weight polar compounds such as simple sugars or small polyhydric alcohols (polyols) are very effective at producing a low SCP by interfering with initial seed crystal formation. The depression in SCP caused by a given concentration of polyol or simple sugar is three to four times greater than the depression in the freezing point (Somme, 1982).

A third type of antifreeze mechanism involves an effect called thermal hysteresis, or difference between a solution's freezing point and melting point. Thermal hysteresis is produced by special proteins or glycoproteins that adsorb to incipient ice crystals, inhibiting further spread of ice within the body (DeVries, 1983; Duman, 1979; Duman and Horwath, 1983). Since these proteins inhibit ice crystal growth, they cause the measured freezing point to be several degrees lower than the melting point, and thus are called thermal hysteresis proteins (THPs). When ice crystals finally grow at the lowered freezing point, they form unusual and characteristic needle-like shapes as a result of the protein inhibitor. Unlike supercooling antifreezes, THPs function in the presence of ice crystals, and seem particularly effective at preventing ice crystals encountered in the environment from radiating by inoculative freezing across the skin or exoskeleton into body fluids (DeVries, 1971; DeVries, 1980; Duman et al., 1982; Tsutsui et al., 1988).

The first and most common cryoprotectant reported in insects was glycerol (Salt, 1957; 1959; 1961; Somme, 1982; Wyatt and Kalf, 1958). Glycerol's cryoprotective properties were first discovered by researchers attempting to preserve spermatozoa (Polge et al., 1949). Other polyhydroxy alcohols (polyols), such as sorbitol and mannitol, also are associated with insect cold-hardiness (Mansingh and Steele, 1973; Ring, 1977; Salt, 1957; Somme, 1967; Somme and Block, 1982). Elevations of glucose (Ring, 1977; Tanno, 1964), or trehalose (Asahina and Tanno, 1964; Morrissey and Baust, 1976; Ring, 1977;

Somme and Block, 1982; Tanno, 1964) were shown to be important correlates of supercooling ability in some insects. Even increased concentrations of free amino acids (primarily proline) aid in freeze-protection of some insects and plants (Mansingh, 1967; Morgan and Chippendale, 1983).

Most early reports described single component systems, while many recent studies have found multifactorial cryoprotective systems, in which several components are thought to play a part in freeze-resistance (Mansingh and Smallman, 1972; Miller and Smith, 1975; Morrissey and Baust, 1976; Ring, 1977). The change is probably due to developments in analytical methodologies now applied within the discipline (Baust and Rojas, 1985). The advantage of a multifactorial system may be to reduce the toxic effect of any one component (Ring and Tesar, 1980). Although increased concentrations of these compounds usually are related to increased supercooling ability, the protective effect is also thought to extend to protein and lipid stabilization during temperature fluctuations above freezing (Arakawa and Timasheff, 1982; Fink, 1986), or during freezing (Anchordoguy et al., 1987; Quinn, 1985)

Monarch Cold-hardiness

In the present study of monarch cold-hardiness, I explored four questions: (1) Does the hemolymph of overwintering monarch butterflies exhibit the characteristic thermal hysteresis diagnostic of THPs? These proteins would probably be the optimal antifreeze for climatic conditions at the Mexican sites, as they would prevent external ice (snow, sleet, freezing rain, frost) from radiating into the bodies of exposed monarchs. (2) Do overwintering monarchs have lowered supercooling points compared with those of monarchs tested in the summer and those of other danaid species that do not extend their ranges into northern areas? (3) How do the ecological factors at the overwintering sites affect the cold-survivorship of monarchs, and how does this relate to the anthropogenic changes that are taking place? (4) What are the biochemical correlates of cold-hardiness in overwintering monarch butterflies?

CHAPTER 2
COLD-HARDINESS IN THE ANNUAL CYCLE OF THE MONARCH BUTTERFLY

Introduction

Variations on the physiological themes of cold-hardiness have been found in numerous insects, other poikilotherms and plants, and may have evolved many times. The monarch butterfly is the only one of 157 species of the tropical subfamily Danainae to migrate extensively into the temperate zone (Ackery and Vane-Wright, 1984). Migration is obviously a behavioral means of cold-protection, but physiological cold-hardiness may also be in the monarch's repertoire. Freezing temperatures are not uncommon at the high-altitude overwintering sites, and probably have been a selective force on the monarch genome for some time. Preliminary evidence that the majority of the overwintering butterflies survived an intense winter storm suggested the hypothesis that monarchs might have evolved some mechanism of freeze protection.

In the present chapter I explored two questions: (1) Do overwintering monarchs have thermal hysteresis proteins (THPs)? Exposure to sub-zero temperatures at the overwintering sites tends to be associated with snow, sleet, freezing rain, and/or frost. THPs would seem to be the most efficient cryoprotection system for this environment, since these proteins would prevent external ice from radiating into the butterflies. (2) Do overwintering monarchs have lowered supercooling points compared with summer monarchs and other danaid species that do not extend their ranges into northern areas?

Methods

Measurement of Thermal Hysteresis

Hemolymph from overwintering and migratory monarchs was tested for thermal hysteresis. Each sample of hemolymph (2-3 µl) was obtained by abdominal puncture and drawn into a 10 µl capillary tube. The end away from the sample was flame sealed, and allowed to cool. Then the sample was shaken down into that end, and the open end was sealed by injection of a layer of mineral oil. This end was inserted into a slit in the bottom of a rubber stopper, so that when the stopper was plugged into a small insulated chamber, the fused end (with the sample) projected within. Ethylene glycol from a Haake cold temperature bath (model A80) was circulated through the chamber, and a dissecting microscope was positioned at plexiglass viewports so that crystal growth and shrinkage within the hemolymph sample could be closely observed. Polarizing filters at the viewports allowed a clearer image of the ice crystal. Hemolymph temperature was estimated with a YSI tele-thermometer connected to a small surface thermistor (YSI, 400 series) affixed in the ethylene glycol within 5 mm of the hemolymph sample. After an ice crystal was induced in the hemolymph by spraying the capillary tube with freon spray (Cryoquik, American Scientific), the tube and stopper were quickly inserted into the chamber and the bath temperature raised or lowered at 0.2°C/min. The freezing point was taken as the temperature at which the seed crystal began to grow during temperature decrease, and the melting point was the temperature at which the seed crystal began to melt during temperature increase (DeVries, 1971; DeVries, 1983; Duman and Horwath, 1983).

Measurement of Supercooling

For determination of the supercooling points (SCPs) of live monarchs, the Haake cooling bath lowered the temperature of the ethylene glycol solution from just above zero to -25°C, at a rate of 0.44°C/min. During this slow drop in temperature, several 25 ml vials (six or seven at a time), each plugged with a rubber stopper and containing a live monarch, were submerged (except for the top cm) in the bath liquid. A small YSI surface thermistor was secured with masking tape to the anterior ventral surface of the abdomen, and body temperatures were recorded at one minute intervals using the YSI tele-thermometer. I previously determined that this measurement of body temperature was identical to that taken within the thorax by a needle thermistor (YSI series 500, 34 gauge hypodermic probe). The monarchs, then, were not in contact with the bath liquid, but with the air inside the immersed vials (a capillary tube through the stopper allowed pressure equilibrium). When freezing occurred at the SCP, I recorded this point as the lowest body temperature reached before the sharp elevation in body temperature caused by release of the latent heat of fusion (as ice radiated within the butterfly) (Salt, 1961; Somme, 1964).

Overwintering Samples

In 1984, SCPs were initially tested in the laboratory at the University of Florida on a small sample of butterflies sent back alive on ice in glassine envelopes from one of the Mexican overwintering sites (Table 2-1, sample 7). Subsequently, on-site studies were deemed necessary because any cold-hardiness mechanism involved might be altered between the time of collection, shipment and analysis. A field laboratory was established, powered by a Honda 3000 watt generator, near the large overwintering site on the Sierra Chincua in Michoacan, Mexico, from January through March, 1985 and 1986. During the winter in 1985, the THP and SCP determinations were run on monarchs collected during four activities, i.e., hanging in clusters, nectaring at flowers, flying, and drinking at streams (Table 2-1, sample 8), whereas in 1986 all were collected from clusters (Table 2-1, sample 9).

Table 2-1. Mean supercooling points (°C), standard deviations and ranges of various samples and species of Danaus butterflies tested. Samples designated A are significantly different from B ($P<0.05$; one factor ANOVA, Scheffé F test).

Sample number	Collection site	Date		Sample size	Condition	Supercooling Point (°C)		Range below zero	Significance grouping
						Mean	SD		
SUMMER									
1. (D. plexippus)	North Florida	July	1986	11	Freshly eclosed	-4.1	1.5	2-7	A
2. (D. plexippus)	South Florida	August	1985	23	Summer fields	-5.1	1.6	1-8	A
3. (D. gilippus)	South Florida	August	1985	49	Summer fields	-4.9	2.4	1-10	A
4. (D. eresimus)	South Florida	August	1985	22	Summer fields	-5.3	2.3	2-10	A
AUTUMN									
5. (D. plexippus)	North Florida	November	1985	29	Autumn breeders	-4.4	1.5	1-7	A
6. (D. plexippus)	North Florida	October	1985	41	Autumn migrants	-7.6	2.1	2-13	B
WINTER									
7. (D. plexippus)	Mexico	January	1984	8	Winter, clusters	-8.2	0.8	4-10 .	B
8. (D. plexippus)	Mexico	Jan-Mar	1985	407	Winter, various activities	-7.7	2.5	1-14	B
9 .(D. plexippus)	Mexico	Jan-Mar	1986	412	Winter, clusters	-8.2	2.4	2-13	B
SPRING									
10 (D. plexippus)	North Florida	April	1984	7	Spring migrants	-6.6	0.9	5-8	AB

Spring, Summer and Autumn Samples

To compare the cold-hardiness of overwintering monarchs with that of summer monarchs and two other Danaine species, SCP tests were done on several further groups of butterflies (Table 2-1). One comparison was of freshly eclosed monarchs tested in July, 1986 in Gainesville, Florida (Table 2-1, sample 1). These butterflies (from eggs laid by monarchs captured in north Florida) were raised in the laboratory on Asclepias curassavica at about 20°C, exposed to natural photoperiods, and tested 24 hours after emergence. The next group consisted of migratory monarchs captured on the Gulf Coast of northern Florida at Cape San Blas in April 1984 (Table 2-1, sample 10) and at St. Mark's National Wildlife Refuge in October 1985 (Table 2-1, sample 6). Both samples were brought back to the laboratory and tested for SCP within 7 hours of capture. In November 1985 a sample of reproductively active, migrant monarchs was collected from an experimental milkweed patch (Asclepias curassavica) at the University of Florida (Table 2-1, sample 5). I also tested a group of reproductively active, non-migrant butterflies in South Florida. Monarchs and two closely related Danaine species, Danaus gilippus berenice and Danaus eresimus (Table 2-1, samples 2, 3, and 4) were collected in August 1985 from several areas immediately west of Miami, Florida. All butterflies were kept in glassine envelopes over ice in a cooler until testing; SCPs were determined within 7 hours of capture.

Lethality of the Freezing Event

Until recently, it has been assumed in SCP studies that the subject remained alive during cooling until the actual freezing event occurred. Knight et al. (1986), however, showed that pre-freezing mortality occurs in some insects before the SCP is recorded. In testing adult grain aphids Sitobion avenae at a cooling rate of 1°C/min, they measured a mean supercooling point of -24.2°C. However, mortality was observed at -5°C and none of the aphids survived temperatures of -15°C. In order to check for pre-freeze mortality in the present study, 15 control monarchs were removed from the cooling bath at temperatures below -10°C before their SCP occurred and then placed in nylon mesh cages for 24 hours at ambient temperatures. Their survival was compared with that of 70 monarchs which had been cooled until SCPs were recorded and also subsequently held in observation cages for 24 hours.

Results

Thermal Hysteresis

Freezing and melting points of hemolymph from 20 overwintering monarchs in Mexico and of 10 autumn migrants in Florida are given in Table 2-2. Although there is a significant difference between the means of freezing and melting points in both the overwintering and migrant samples, this difference is very small, less than 0.1°C in both comparisons. Formation of needle-like crystals characteristic of solutions containing THPs did not occur during the growth of these crystals.

Supercooling

A summary of mean SCPs of the different samples is given in Table 2-1. Since there were no significant differences in SCP with regard to sex in any of the samples (one factor ANOVA), both sexes were lumped in further analyses. The distributions of supercooling points in all samples tested were normal (Kolmogornov-Smirnov tests for normality, two-tailed, all >0.05) and shifted from higher SCPs in reproductive to lower SCPs in migratory and overwintering monarchs (Figure 2-1). Sample means were compared using a one factor ANOVA and Scheffé F test. The mean SCPs of overwintering samples were all significantly lower than mean SCPs of reproductively active monarchs and closely related Danaine butterflies (Table 2-1). Autumn migrants also

Table 2-2. Melting points (A), freezing points (B), and thermal hystereses (A-B) of hemolymph (°C) overwintering season in Mexico (means ± SD). The differences between mean melting and mean freezing points were analyzed by paired t-tests.

		A	B	A-B	A vs B	
	N	Melting point	Freezing point	Thermal hysteresis	Paired t-value	P
Autumn (Florida)	20	-0.92 ± 0.04	-0.98 ± 0.06	0.06 ± 0.05	3.67	0.005
Winter (Mexico)	10	-0.94 ± 0.05	-1.02 ± 0.05	0.07 ± 0.07	4.68	0.0002

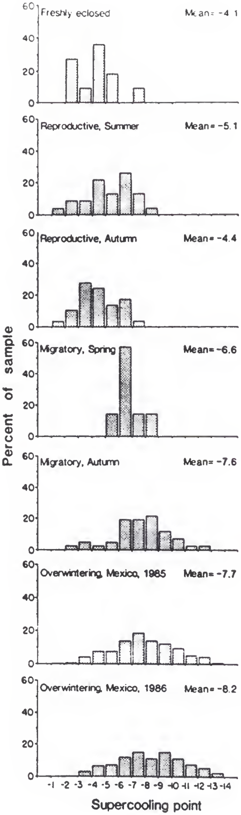

Figure 2-1. Frequency distributions of supercooling points for samples of monarch butterflies during various phases of their annual cycle. See Table 2-1 for more details. Samples of freshly ecolsed, summer reproductive, autumn reproductive, spring migrants, autumn migrants, and overwintering monarchs from Mexico in 1985 and 1986 are samples 1, 2, 5, 6, 7, 9, and 10, respectively, of Table 2-1.

had significantly lower SCPs than monarchs that were breeding in North Florida in the fall. The seasonal changes in monarch SCP are summarized in Figure 2-2.

SCP and Activity

Mean SCPs of overwintering butterflies caught flying, nectaring or drinking were not different from each other, but were significantly different from the mean SCP of clustering butterflies (Table 2-3).

SCP and Lethality

In Mexico, all 15 clustering butterflies removed from their cool-down period in the SCP test before freezing showed complete recovery within an hour in the nylon cages at ambient temperature with no observable ill effects. These monarchs had experienced body temperatures of -10 to -12°C during the test. Another 70 monarchs taken through the same cool-down period and showing evidence of freezing at their SCP were subsequently observed for 24 hours under the same ambient conditions. None recovered.

Discussion

The experiments reflect an annual change in monarch supercooling ability, with autumn migrants and overwintering samples freezing lower than those in the summertime reproductive state. Supercooling points of the summer monarchs were not statistically different from SCPs of two close congeners, D. gilippus and D. eresimus, collected and tested at the same time in south Florida. At the Mexican overwintering sites, clustering monarchs were more resistant to freezing than were monarchs engaged in other activities. Freezing, not cooling, was found to be the lethal event.

Although a THP mechanism would probably be very effective under the high-altitude winter conditions, I found only a slight thermal hysteresis, less than 0.1°C, in

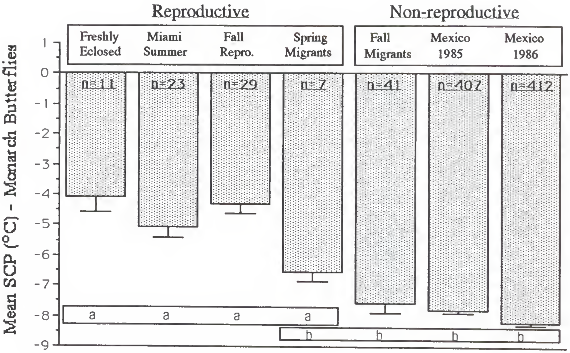

Figure 2-2. Seasonal changes in supercooling point (SCP) shown by the monarch butterfly during various parts of its annual cycle. Samples designated 'a' are significantly different than samples 'b' (one way ANOVA, Scheffe F test). Bars = Standard error.

Table 2-3. Mean supercooling points (°C), standard deviations and ranges of overwintering monarchs in Mexico, 1985, collected during four different activities (subset of Sample 9, Table 1). Clustering monarchs had significantly lower SCPs than the other three samples ($P<0.05$; One factor ANOVA, Scheffé F test).

		Supercooling point			
Activity	N	Mean	SD	Range	Test group
Clustering	157	-8.9	2.3	-3.5 to -14.0	A
Nectaring	40	-7.6	2.0	-1.5 to -12.0	B
Flying	28	-7.2	2.1	-4.2 to -12.9	B
Drinking	123	-6.5	2.2	-2.5 to -12.8	B

monarch hemolymph (Table 2-2). In other studies where THPs have been found, the thermal hysteresis was much greater, ranging from 2.2 to 5.6°C (Duman et al., 1982), and characteristic crystal spires were reported. These crystal shapes did not occur in my experiments. I conclude that, while there was a barely measurable hysteresis, evidence for functional THPs in overwintering monarch butterflies is lacking at this time.

The observed supercooling ability in monarchs may be adequate for normal overwintering conditions in Mexico, but it is moderate compared with the supercooling ability of some non-migrant insect species that inhabit boreal climates. Many of these spend the winter as eggs or larvae that can withstand ultra-low temperatures. Mean SCPs as low as -51°C were found in eggs of the tortricid Zeiraphera diniana from inland Norway (Bakke, 1969), and eggs of the aphid Pterocomma smithia from Alberta could survive to -42°C (Somme, 1964). In larvae of the bark beetle Scolytus multistriatus, a mean SCP of -53°C was recorded (Lozina-Lozinskii, 1974), and gall forming midge larvae (Rhabdophaga sp.) from the North Western Territories had a mean SCP as low as -61°C (Ring and Tesar, 1980). In contrast, insects that overwinter as adults are much less cold resistant, with only a few having mean SCPs below -25°C. There are three reports of adult butterfly cold-hardiness, all in the Nymphalinae. In interior Alaska, mourning cloaks (Nymphalis antiopa) and a species of Polygonia had mean SCPs of -20 and -25°C, respectively (Miller, 1982). Vanessa urticae from Leningrad showed a mean SCP of -21°C (Lozina-Lozinskii, 1974). These boreal butterflies are understandably more cold-protected than monarchs, in relation to past selection pressures of colder temperatures. Enduring the harsh winter *in situ* is part of an evolutionary strategy that allows extended access to favorable resources. This strategy also seems to relate to the type of host plant utilized; butterflies that overwinter as adults in northern areas are frequently associated with woody host plants (Hayes, 1982). The alternate tactic of the monarch, migration over great distances, probably evolved primarily in relation to the availability pattern of its (herbaceous) milkweed hosts (Brower, 1985; Cockrell et al., In Press; Malcolm et al., In Press).

Avoidance of severe northern cold may be of secondary importance. My results suggest that there is a potential for the evolution of more extreme cold-hardiness in the natural variation in monarch SCP, depending on the heritability of this variation.

If this moderate supercooling ability of the monarch is in fact a physiological adaptation to overwintering in Mexico, it must relate to current and past minimum temperatures at the altitude and latitude of the current overwintering sites. All major monarch colonies have been found within an area of about 7000 km^2 (between 19°10' and 20°00'N latitude and 99°55' and 100°40'W longitude) at an average altitude of 3,000 m (Calvert and Brower, 1986; Calvert et al., 1989). While there are no temperature data from overwintering sites before their discovery in 1974, records are available from several high altitude weather stations located within the 7000 km^2 area. Table 2-4 shows average minimum and extreme minimum temperatures over 10-26 years from nine weather stations in this area above 2,500 m during January, the coldest month. The average lows of January for these stations ranged from 2.4 to -1.7°C, and the extreme lows ranged from -5 to -14°C. A temperature as low as -14°C would kill virtually all overwintering monarchs (Figure 2-1). However, weather stations are usually situated in clearings, which are colder and less protected than within the forest where monarchs cluster (Calvert and Brower, 1981; Calvert et al., 1989; Calvert et al., 1982).

The monarch migration/diapause syndrome is a physiological state cued by environmental stimuli, mediated by endocrine mechanisms and involving several behavioral and physiological changes in the adult butterflies (Herman, 1985; Herman et al., 1981; Tauber et al., 1986). Some of these changes are cessation of reproduction, migratory behavior, lipid storage and low levels of juvenile hormone (Herman, 1985; Lessman and Herman, 1983). In the present study, autumn migrants show increased cold-hardiness, but reproductively active monarchs in autumn have the same SCP as reproductively active summer samples. These autumn reproductives were probably migrants that had broken diapause in response to warm temperatures and encounters with our large garden of

Table 2-4. Average January minimum and extreme low temperatures (°C) recorded at nine, high-altitude weather stations near the monarch overwintering sites in the states of Mexico and Michoacan. 10-26 years of data (Anon., 1982).

Weather Station	Latitude (°N)	Longitude (°W)	Altitude (m)	Average January low	Extreme minimum
MEXICO					
Almologa del Rio	19°09'	99°29'	2,670	-1.7	-9.0
Atlacomulco	19°48'	99°53'	2,526	1.5	-9.0
Rio Frio	19°21'	98°40'	3,000	-0.3	-14.0
San Felipe del Progresso	19°42'	99°58'	2,550	2.0	-9.0
Tenango del Valle	19°06'	99°35'	2,660	1.1	-9.0
Texcoco	19°33'	98°53'	2,800	-0.5	-11.5
MICHOACAN					
Villa Victoria	19°27'	99°59'	2,608	-0.7	-8.9
Presa Pucuato	19°38'	100°42'	2,505	2.0	-6.0
Presa Sabaneta	19°38'	100°41'	2,515	2.4	-5.0

cultivated milkweed plants at the University of Florida. If low juvenile hormone titers mediate the migration/diapause state, and high JH levels lead to reproduction (Herman, 1985), then the seasonal changes in SCP shown in this study probably reflect a cold-hardiness physiology also influenced by JH levels. This leads to the testable prediction that injection of JH will not only cause overwintering monarchs to break diapause, as Herman found, but result in higher SCPs. This experiment was performed in 1986 and is reported in Chapter 3, along with the discovery that SCPs of overwintering monarchs show a circadian fluctuation.

CHAPTER 3
A CIRCADIAN FLUCTUATION OF FREEZE RESISTANCE IN OVERWINTERING MONARCH BUTTERFLIES IN MEXICO

Introduction

Insect cold-hardiness is generally thought to involve slow, seasonal changes in response to reliable environmental cues. Such seasonal cold-hardiness has been related to gradual changes in concentrations of metabolic cryoprotectants (such as polyols, sugars, and thermal-hysteresis proteins) and/or ice nucleating agents (Baust, 1982; Duman et al., 1982; James and Luff, 1982; Miller and Smith, 1975; Rains and Dimock, 1978; Salt, 1961; Shimaka et al., 1984; Smith, 1961; Somme, 1982; Takehara, 1966; Zachariassen, 1980). However, a more rapid cold-hardening change in several non-diapausing insects has recently been discovered (Lee et al., 1988). Exposure of larvae and pharate adults of the flesh fly, Sarcophaga crassipalpis, adults of the elm leaf beetle, Xanthogaleruca luteola, and the milkweed bug, Oncopeltus fasciatus, to short term chilling at 0°C was found to confer protection against cold shock during subsequent exposure to sub-zero temperatures. This unusual finding of a quick shift in cold-hardiness may be anomalous, or it may be indicative of a more widespread but overlooked capacity for faster response. Here I report another finding of rapid changes in insect cold-hardiness: a circadian fluctuation in the freeze-resistance of overwintering monarch butterflies (Danaus plexippus, L.) in Mexico.

Early the second winter (1986) at the overwintering site on the Sierra Chinqua, I began to suspect and explore the possibility that monarch supercooling points were lower in the morning and at night than during the day. Experiments were designed to (1) quantify the daily change in SCP, (2) determine if the circadian change was free-running or cued by external stimuli, and (3) investigate the influence of methoprene, a JH analogue, on SCP.

Methods

During the months of January through March in 1985 and 1986 a field laboratory was maintained at the large monarch overwintering site on the Sierra Chincua near Angangueo, Michoacan, Mexico. Supercooling points (SCP) of freshly collected monarchs were measured as described in the Methods section of Chapter 2.

Daily Change in SCP

To control for extraneous variables such as activity or differential intake of nectar and water, I collected monarchs for each day's experimentation either early in the morning or late the previous evening (after all flight had ceased for the day) and kept these butterflies in nylon mesh cages under ambient, partially shaded conditions. Throughout the day, samples were randomly selected from these cages and tested for SCP.

Light and Temperature

An attempt was made to isolate some of the monarchs from light and temperature cues, to see what effect, if any, this would have on the daily SCPs. At night, I placed freshly collected monarchs in glassine envelopes within perforated jars that were wrapped in aluminum foil, put inside dark plastic bags and suspended in ice water within a large cooler. Throughout the next day, I retrieved the jars at intervals and immediately tested the monarchs for SCP. In each jar were six butterflies, the number that could be simultaneously assayed, so all jars were in darkness at 0°C until just before testing. Control samples, taken from the mesh cages under ambient conditions, were alternately tested through each day of this experiment, which was carried on for three days.

Hormonal Influence

I also investigated the possible influence of methoprene, an insect juvenile hormone (JH) analogue, on the cold-hardiness of the overwintering butterflies. High JH concentrations in the monarch are known to relate to reproduction, and diapause is associated with very low levels of JH (Lessman and Herman, 1983). If monarch cold-hardiness is a part of the physiogical state of diapause, then treatment with JH (or methoprene) might not only cause overwintering monarchs to break diapause (Herman, 1985), but result in elevated SCPs for treated butterflies. In order to evoke an immediate response, I topically applied a dosage of 200 µg methoprene in 5 µl acetone 30 minutes prior to SCP testing, twice the dosage of JH used in experimental interruption of monarch diapause (Herman, 1981; Herman, 1985). For this experiment (as in the other assays to quantify the daily change in SCP), butterflies were collected from clusters early in the morning or after quiescence the night before, and kept outside through each day in mesh cages until testing. These assays were continued over several days, in alternation with assays of control monarchs treated only with 5 µl acetone.

Results

Daily Change in SCP

Through the winter in 1986, 412 monarchs were tested for supercooling point at various times of day. These SCPs are shown as hourly means in Figure 3-1. As an *a posteriori* test of the apparent cyclicity, the data were grouped for four intervals of daytime (Morning, 7-10; Midday, 10-13; Afternoon, 13-17; and Night, 17-22) and compared using a Student Neuman Keuls ANOVA (Table 3-1). The mean SCP of the midday group is significantly higher than the morning, afternoon and night means ($P < 0.005$).

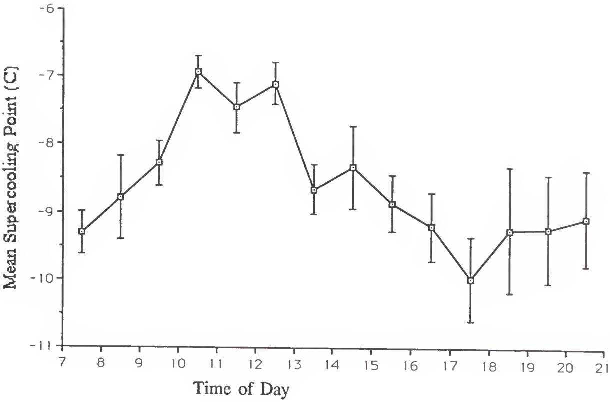

Figure 3-1. Mean hourly supercooling points of 412 monarch butterflies tested at various times of day during Jan.- Mar., 1986. Bars = standard error. These data are given in Table 3-1, pooled for four daytime intervals, with results of statistical analysis.

Table 3-1. Mean supercooling points (SCP) of overwintering monarch butterflies in Mexico tested at different times of day. Experiments were run over a period of 42 days, from Jan. 14 to Feb. 25, 1986. Samples designated A are significantly different from B (one way ANOVA, Scheffe F test, $P < 0.001$).

Time	Hours	N	SCP(°C)	SD	ANOVA
Morning	7-10	90	-9.0	2.2	A
Midday	10-13	167	-7.1	2.1	B
Afternoon	13-17	102	-8.7	2.5	A
Night	17-22	54	-9.3	2.4	A

Light and Temperature

Figure 3-2 shows three days of SCP assays on monarchs kept in the dark at 0°C (referred to as cue-less), compared with SCPs of controls taken from ambient holding cages during the same period. Each point represents the mean SCP (± S.E.) of a sample of six monarchs assayed simultaneously. These data were pooled for four daytime intervals, as before (Table 3-2), and two comparisons were considered. First, mean SCPs from different daytime intervals within each treatment group were compared with one way ANOVA and Scheffe F test (Table 3-2). In controls, just as in the larger sample above, the morning, afternoon and night means were not different, but the midday mean was significantly higher than all of them ($P < 0.0005$). For cue-less samples, the morning, midday and afternoon means did not differ from each other, but the night mean was significantly lower than the other means($P < 0.05$). In a second type of comparison, SCPs of control groups were compared with SCPs of cue-less groups from similar time intervals with one way ANOVA. Although the shape of the daily curve (Figure 3-2) appears flattened in the cue-less samples compared with the curve of controls, the difference is not significant at $P \leq 0.05$ (Table 3-2).

Hormonal Influence

Figure 3-3 shows mean hourly SCPs of monarchs treated with methoprene 30 minutes prior to testing as contrasted with control monarchs sampled from ambient holding cages. As before, data were lumped for four sections of day (Table 3-3) and one way ANOVA was used to compare treatment vs. control SCPs within the same time sector. Morning and midday treatment groups did not differ from controls, but SCPs of methoprene treated monarchs in afternoon and night groups were both significantly higher than controls.

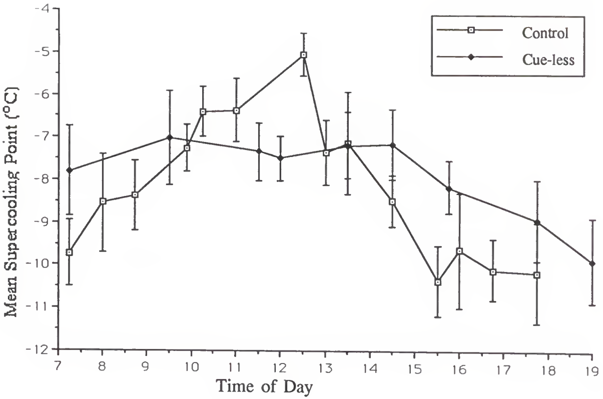

Figure 3-2. Mean supercooling points, tested at different times of day, of samples of monarch butterflies (six per sample) isolated from light and temperature cues (Cue-less) compared with controls from ambient holding cages. Bars = standard error. These data are given in Table 3-2, pooled for four daytime intervals, with results of statistical analysis.

Table 3-2. Mean supercooling points (SCP) tested at different times of day, with sample sizes and standard deviations, of overwintering monarch butterflies isolated from light and temperature cues (Cue-less) compared with controls from ambient holding cages. Experiments were run from 2/8 - 2/11, 1986. Samples within each treatment group designated A are significantly different from B (one way ANOVA, Scheffe F test, P < 0.05) No differences were found in comparisons between treatment groups.

	Cue-less				Control			
Time	N	SCP	SD	ANOVA	N	SCP	SD	ANOVA
Morning (7-10)	12	-7.4	2.1	A	24	-8.5	2.1	A
Midday (10-13)	12	-7.4	1.4	A	23	-6.4	1.7	B
Afternoon (13-17)	18	-7.5	1.8	A	18	-9.0	3.0	A
Late (17-22)	12	-9.4	2.3	B	6	-10.2	2.8	A

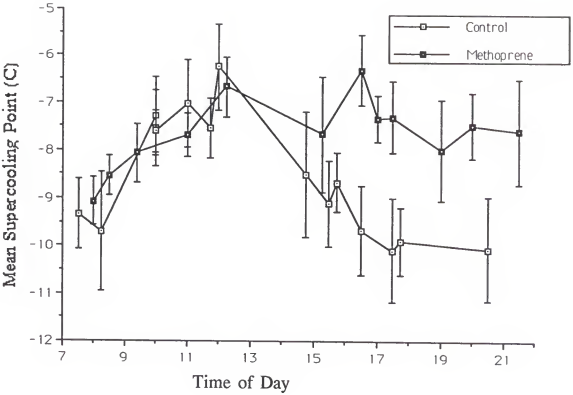

Figure 3-3. Mean supercooling points, tested at different times of day, of samples of monarch butterflies (six per sample) treated with methoprene 30 min. prior to test, compared with controls. Bars = standard error. These data are given in Table 3-3, pooled for four daytime intervals, with results of statistical analysis.

Table 3-3. Mean supercooling points (SCP, °C) tested at different times of day, with sample sizes and standard deviations, of overwintering monarch butterflies topically treated with methoprene in acetone compared with controls treated with acetone. All treatments were 30 min. prior to SCP test. Experiments were run from 1/28 to 2/19, 1986. SCPs of methoprene treated monarchs differed from controls in afternoon and late groups, but not in morning or midday (one way ANOVA, Scheffe F test).

	Methoprene			Control			
Time	N	SCP	SD	N	SCP	SD	Significant Difference
Morning (7-10)	19	-8.5	1.3	29	-8.2	2.3	None
Midday (10-13)	12	-7.2	1.4	18	-6.9	2.1	None
Afternoon (13-17)	18	-7.1	2.0	24	-9.0	2.2	P = 0.008
Night (17-22)	30	-7.5	2.1	24	-10.0	2.2	P = 0.0001

Discussion

Daily SCP Change and Diapause

This is the first reported circadian change in cold-hardiness in insects. My evidence indicates that it is hormonally influenced, and is probably part of the overwintering diapause state. Diapause in insects, once simply referring to dormancy, is now understood in a more broad sense to mean a syndrome of physiological and behavioral programs that underlie several interrelated ecological manifestations (dormancy, seasonal migration, seasonal polyphenism), e.g., Tauber, et al. (1986). In the monarch, juvenile hormone (JH) clearly plays a critical role in the regulation of ovarian growth, male and female reproductive tract development, and diapause (Herman, 1985; Lessman and Herman, 1983). High levels of JH are associated with reproduction, while diapausing adults have very low titers, a pattern also found in two other well studied insects, the Colorado potato beetle, Leptinotarsa decemlineata (de Kort et al., 1982; de Wilde, 1983) and the milkweed bug, Oncopeltus fasciatus (Rankin, 1978; Rankin and Riddiford, 1978). If monarch cold-hardiness is a part of the diapause syndrome, then the prediction follows that treatment of overwintering monarchs with JH (or the JH analogue, methoprene) would not only cause them to break diapause (Herman, 1985) but result in higher SCPs. This prediction was confirmed in the study of methoprene treated monarchs tested in the afternoon and evening. A clear elevation of SCP was observed in these samples compared with controls. However, SCPs of treated monarchs in the morning and midday were not different from those of controls, a result that can not be explained at present; perhaps more research into the underlying hormonal mechanisms in diapausing monarchs will clarify this finding.

Only two other investigations have shown a link between endocrine activity and cold-hardiness in insects. The first (Horwath and Duman, 1983) found that larvae of the beetle Dendroides canadensis accumulate protein antifreezes during the winter. When these larvae were collected in the fall prior to any cold hardening and were treated with topically applied juvenile hormone (in acetone), they developed elevated levels of antifreeze proteins in their hemolymph compared with controls. The second study (Tsumuki and Kanehisa, 1981) found glycerol production to be induced in the larval rice stem borer Chilo suppressalis by treatment with JH and JH analogues. Also, decreased glycerol levels resulted from ecdysone injections. The authors did not measure freeze resistance as such, but logically assumed that this was associated with glycerol concentrations. In both of these studies high JH levels are related to diapause and/or cold-hardiness. However, in adult insects, as previously discussed, low JH levels are usually found in the diapause state.

Daily SCP change: possible endogenous rhythm

The results of the attempt at isolating monarchs from light and temperature cues are inconclusive, and are reported only as preliminary findings. It is interesting, however, that even when monarchs were kept in the dark at 0°C, a change in SCP was seen between samples done in the day vs. those done after 1700. However, this experiment needs to be repeated using an environmental chamber for more precise control of external cues. If under such conditions the SCP does indeed continue in a daily cycle, an endogenous rhythm is indicated.

Daily cycles of different types have been found in a great many organisms, including glycolysis in yeasts (Hastings and Schweiger, 1976), bioluminescence in dinofolagellates, and photosynthesis in plants (Sweeney, 1969), as well as the biological clocks known to function in birds, insects and mammals (Follett, 1982). Aspects of human physiology also cycle, e.g., body temperature, urine metabolites and alertness (Conroy and Mills, 1970). Commonly, circadian rhythms tend to persist in the laboratory under constant conditions, implicating an endogenous origin. However, light and other environmental cues (Zeitgebers) usually function to entrain or re-set the self-sustained oscillations.

Adaptive significance

Overall, monarchs diapausing in the winter in Mexico have moderately lower supercooling points than when they are reproducing in the north in summer. In addition, this seasonal increase in freeze resistance is more pronounced at night. Both of these experimental observations seem to point to an adaptive pattern of physiology that plausibly results from past selection pressures at the high-altitude overwintering sites. A major climatic pattern of this and other tropical, high-altitude environments is an extreme diurnal temperature fluctuation, especially on clear days (Smith and Young, 1987).

At the monarch overwintering sites, an increase in cold-resistance at night would seem very adaptive. These areas, at an average elevation of approximately 3000 m, are subject to intense insolation by day and rapid radiational cooling at night. Because ambient air temperatures plummet after dark, and butterflies exposed to the night sky cool down as much as 4°C below air temperature (see Chapter 4), nighttime poses a special threat of death by freezing (Calvert and Cohen, 1983). In the day, the ability to warm to flight threshold by basking in order to regain position within the clusters or fly to water or nectar sources is probably essential for survival (Calvert and Brower, 1986; Masters et al., 1988). Any physiology that produces supercooling, presumably based on increased hemolymph concentration of some metabolite, might interfere with this capacity for mobility during the day. Detrimental effects of high concentrations of cryoprotectants have been suggested (Fahy, 1986) and it is possible that there are 'costs' associated with such antifreezes that must be balanced against the protection afforded.

Some other monarch characteristics also seem particularly adaptive to such a daily temperature flux. The thermoregulatory behaviors (Masters et al., 1988) make the monarch adept at coping with the rapid warming and cooling shifts inherent at high elevations, and at conservation of lipids under these conditions. The crawling-up behaviors (Alonso et al., In Press) move monarchs off the ground if they are trapped there by rapidly declining temperatures at sunset. Monarchs that do not escape the ground at night are much more likely to die from rodent attack (Glendinning et al., In Press), or freezing (Calvert and Brower, 1986; Calvert and Cohen, 1983). The dramatic flight response by large numbers of butterflies when a cloud obscures the sun is probably also an escape from being grounded by nightfall. Considering that the gene pool of eastern U.S. monarchs has convened each winter in this environment for perhaps millennia, it is not surprising to see fine-tuned adaptive characteristics.

Other organisms living in tropical high-altitude environments exhibit many aspects of morphology and physiology that seem to provide escape from, or tolerance to, extreme daily temperature change. In many montane areas of the tropics, plants show recurrent convergence on a giant rosette form, which is thought to represent a syndrome of interrelated physiological and morphological characters that buffer the plants against extreme diurnal environmental variations (Smith and Young, 1987). Most of these plants are very cold-hardy, and can tolerate freezing or have supercooling capacity. One species, Polylepis sericea (Rosaceae), a common treeline plant in the northern Andes, has even been found to have daily changes in supercooling ability of the leaves. The supercooling point of the cell sap is lower at night (Goldstein et al., 1987).

Slow seasonal changes in cold-hardiness are undoubtedly widespread in plants and poikilothermic animals, but rapid changes may be more prevalent than previously suspected. In deserts and at high altitudes, organisms subject to large daily temperature fluctuations are perhaps on the horns of a selective dilemma, requiring activity during the day and freeze-resistance at night. The evolution of a daily cycle of cold-resistance seems a resolution to this problem.

CHAPTER 4
ECOLOGICAL FACTORS CRITICAL TO THE COLD-HARDINESS OF OVERWINTERING MONARCH BUTTERFLIES IN MEXICO

Introduction

After ages of evolution, organisms now possess a fascinating array of adaptations that function for life support and reproduction in particular environments. However, the result of natural selection is adequacy rather than perfection. Limitations are inevitable from constraints of phylogenetic history and compromises resulting from numerous, disparate selection pressures. Also, adaptations were actually engendered by past situations, and their effectiveness is usually lessened by environmental change, especially when it is rapid (Bennett, 1987). This last consideration is especially important in our present age of widespread anthropogenic change. A complete study of any adaptation, therefore, must proceed to understand not only how the mechanism operates, but how its success is limited by ecological context.

Monarch Cold-hardiness

A case in point is the monarch butterfly (Danaus plexippus, L.). In controlled, experimental studies, I found that monarchs overwintering at high-altitude Mexican sites have a moderate, seasonal increase in supercooling ability compared with reproductive monarchs or congeners from Florida. Interestingly, the supercooling points (SCP) of overwintering monarchs fluctuate on a daily basis, so that they are able to survive lower temperatures at night than in the day. This pattern of cold-hardiness seems adaptive in a tropical alpine environment (Smith and Young, 1987), where large daily swings of temperature occur under clear skies. The freeze-resistance achieved is only moderate compared with more boreal insects; but it appears to be adequate under most winter conditions.

The test for supercooling point (SCP) is an established cryobiological assay (Salt, 1961; Somme, 1982). The temperature was recorded at which the whole butterfly spontaneously froze when cooled at a slow, standard rate. This standard rate of cooling was necessary for comparative purposes, but different rates of cooling might result in different SCPs (Baust and Rojas, 1985). The results, therefore, are lethal limits only under the controlled conditions of the test. I also suspected that these lethal limits were affected by the common environmental conditions at the overwintering sites, and designed experiments to quantify the effects of two major ecological factors on monarch cold-hardiness.

Environmental Factors — Wetting

Surface water on an insect (or plant) tends to freeze at relatively high temperatures, and ice crystals can radiate into the body by innoculative freezing, unless blocked by integument or inhibited by some internal factor (Block, 1982a; Duman et al., 1982; Somme and Conradi-Larsen, 1979). The episodes of sub-zero temperature at the Mexican overwintering sites usually occur during storms associated with rain and snow. Also, exposed surfaces at night commonly become wet with dew. I carried out experiments at the overwintering site in 1985 to determine the effect of wetting on cold-hardiness and the role of overhead canopy cover in protection from wetting.

Environmental Factors — Exposure

Incoming solar radiation is absorbed by objects depending on their albedo or reflectivity. This absorbed energy is then re-radiated in the form of longer wavelength radiation. At night this emission continues without the sun's input. If the object in consideration is under cloud cover, tree canopy, etc., much of the thermal radiation re-emitted is bounced back from the cover and surroundings and regained, but if the cold night sky is overhead much radiation leaks directly to the absolute zero of outer space. This leads to a cooling effect: the temperature of the object falls and comes into equilibrium below ambient air temperature, depending on amount of exposure, air temperature, and temperature of substrate (Geiger, 1980). If relative humidity is high, condensation and frost can result on the colder surfaces, even if ambient air temperatures do not go below freezing. An exposure experiment was constructed in 1985 at the overwintering site to find out exactly how the monarchs were affected by exposure to the night sky.

Methods

Experimental Measurement of Supercooling Point

During the months of January through March in 1985 and 1986, we set up a field laboratory at the large monarch overwintering site on the Sierra Chincua near Angangueo, Michoacan, Mexico. The supercooling point (SCP) measurements were determined there as described in Methods, Chapter 2.

Wetting — Effects of Rain

The first rain of 1985 at the overwintering site fell on Feb. 27 and lasted for 75 minutes, ending about 18:30. The wind blew the rain lightly from the north-east (from up-slope), as was the case in the three rainfalls that followed in March. Immediately following the rain, butterflies were collected from different positions of exposure within the monarch colony: from surface locations and from within both large and small bough clusters, from trunk clusters on the north and south sides of trees, and from ground vegetation in a small clearing. Ten butterflies from each position were plucked with large forceps and put in labeled zip-lock bags (one per position) for transport to camp. Half of the monarchs from each bag were immediately tested for supercooling point, then numbered and put in a nylon mesh cage to dry. The remaining 5 monarchs from each bag were numbered and allowed to dry in another cage for 24 hours, after which time they were tested in the same manner for SCP. All butterflies and bags were weighed with an electronic balance just after the rain and again 24 hours later after drying. This experiment, then, allowed a determination of the relationship between position within the colony, wetting, and freeze-vulnerability. At the time the butterflies were sampled from the monarch colony, movement had ceased until the next morning. If ambient temperatures had fallen to dangerous lows after the rain (as sometimes happens in winter storms), the pattern of mortality found in the experimental test would have been reflected in the colony.

Wetting — Effects of Controlled Spraying

I also ran SCP tests on butterflies that had been sprayed with a fine mist of water from a hand-operated garden sprayer. Freshly collected monarchs were weighed, sprayed with from one to nine sprays, weighed again and tested for SCP. In this experiment, the amount of surface water on individual monarchs could be related to their SCP.

The Overhead Canopy — Protection from Wetting?

To investigate the relationship between tree cover and wetting, I measured rainfall at 71 different points within the monarch colony during each of three March rains in 1985. A bicoordinate grid of 10 by 10 meter quadrats was established within the colony early in January, 1985. According to a subjective estimation of canopy thickness overhead, each quadrat was ranked on a scale of 1-5, with 1 designating open or completely exposed to the sky, and 5 as most dense canopy. Rainfall monitoring points (styrafoam cups, 8 cm in diameter at the opening, stapled on top of stakes 1 meter high) were set up in the center of all quadrats except those ranked 3 (or medium coverage). Cups were also attached to the northeast and southwest sides of seven large and seven small trees within the colony. Immediately after each of the three rains of March, I measured the volume of water in each cup with a calibrated plastic syringe fitted with a rubber tube. Quantification of canopy cover at each quadrat site was obtained by photography: a 35 mm Leica with a 12 mm wide angle lens was placed on a leveled platform on each stake for an overhead photograph. Resultant negatives were later projected onto an Apple computer graphics tablet and the open areas of sky were traced and calculated, providing an objective determination of overhead exposure.

Exposure — The Effect of Radiational Cooling

An experiment was run in Feb. and March, 1985 to examine the relationship between monarch body temperature and degree of exposure to the night sky. In a wide clearing close to the campsite, I suspended four large squares of greenhouse cloth horizontally about 0.5 m above the ground. Each was a square meter of woven, plastic mesh screen with different shade qualities obtained from Seminole Stores, Gainesville, FL. The exact shading produced by each screen was quantified using a Ly-Cor pyranometer sensor with an LI-integrator. The quantity of sunlight reaching the sensor through each screen was divided by the average value of full sunlight measured immediately before and after the screen measurement. These values (x 100) came to 5, 26, 51, and 72% exposure through each screen. Wooden planks 20 cm wide were positioned about 50 cm below these screens and approximately 50 cm from the ground. On eleven different nights, I left live monarchs on the planks beneath these artificial covers (as well as under no cover, or 100% exposure) with thermistors taped to their abdomens (as in the SCP tests) for measurement of body temperature. At 04:30 in the morning, I recorded their body temperatures, and ambient air temperature at the level of the butterflies.

Results

Rain, SCP, and Position Within Colony

Results from the rain experiment are shown in Table 4-1. Butterflies sampled from the surface of a cluster or from open vegetation had more surface water and froze at significantly higher temperatures than butterflies collected from within or from the bottom of a cluster ($P < 0.001$, ANOVA, Scheffe F test). These more exposed monarchs also had significantly higher SCPs than other members of their bag that were allowed a day to dry (One way ANOVA, $P < 0.05$). Monarchs collected from the northeast side of the fir were wetter and froze sooner than those on the southwest side; however, those from different sides of the pine showed no difference.

SCP and Amount of Surface Water

When water was added to butterflies by spraying, a significant relationship was found between amount of surface water and SCP (linear regression, $y = 0.04x - 5.95$, $r2 = 0.23$, $P = 0.0001$). Table 4-2 shows the mean values of surface water added and SCPs for samples of monarchs, including controls that were not sprayed. Most of the effect on SCP comes with very small amounts of water, however, and almost any wetting increases the risk of freezing considerably. Figure 4-1 is a comparison between SCPs of wetted monarchs (over 1 mg H_2O on the body) and all other SCPs tested on freshly collected monarchs from clusters in 1985, and in 1986. In the tests, half the wetted butterflies were frozen at -4.18°C, whereas less than 5% of the dry, clustering monarchs were frozen at this point. By -8°C, a temperature at which almost half of the samples from clusters had frozen, all of the wetted monarchs were frozen.

Table 4-1. Mean supercooling points (±SE) of monarch butterflies collected from different locations within the monarch overwintering colony on the Sierra Chincua after a rain (Feb. 27, 1985) compared with those from the same location allowed to dry 24 hours before testing. Each test group = 5 monarchs. One-way ANOVA; differences at $P < 0.05$ are marked with *.

Sample location	mg water per monarch	Mean SCP after rain	Mean SCP after drying	ANOVA (* = $P < 0.05$)	
Large cluster,					
Northeast	64.5	-3.5±0.3	-7.8±0.6	0.0001	*
Southwest	32.2	-3.5±0.8	-8.4±0.6	0.0012	*
Bottom	18.7	-8.6±0.8	-6.5±0.8	0.10	
Inside	19.0	-8.4±0.8	-7.6±1.1	0.57	
Smaller cluster,					
Northeast	72.4	-3.4±0.5	-6.3±0.8	0.0099	*
Southwest	55.4	-3.3±0.3	-8.1±0.6	0.0001	*
Top	27.7	-3.0±0.6	-5.9±0.6	0.005	*
Bottom	14.4	-6.3±1.8	-9.0±0.9	0.199	
Inside	16.4	-7.9±1.0	-8.0±1.7	0.969	
Fir trunk,					
Northeast	57.5	-3.2±0.2	-7.2±1.1	0.004	*
Southwest	22.0	-7.7±0.5	-8.1±1.1	0.065	
Pine trunk,					
Northeast	26.2	-5.6±0.7	-6.9±1.1	0.632	
Southwest	18.0	-8.6±1.3	-8.9±0.5	0.091	
Open vegetation	84.5	-3.1±0.2	-7.5±1.0	0.0014	*

Table 4-2. Mean supercooling points (±SE) of overwintering monarch butterflies at the site on the Sierra Chincua, Feb., 1985, sprayed with water compared with controls not sprayed. There is a significant correlation between water on body surface and SCP (r = 0.48 p = 0.0001).

	Mean water added (mg)	Mean SCP (°C)
Dry (Control) n = 20	0	-7.7 ± 0.6
1 Spray n = 14	4.2 ± 0.7	-5.2 ± 0.5
2 Sprays n = 21	16.0 ± 1.9	-4.5 ± 0.2
3 Sprays n = 14	28.2 ± 1.5	-3.8 ± 0.2
4 Sprays n = 14	36.7 ± 3.3	-3.9 ± 0.2
5 Sprays n = 7	44.6 ± 2.8	-3.7 ± 0.4
9 Sprays n = 14	79.6 ± 4.4	-4.07 ± 0.3

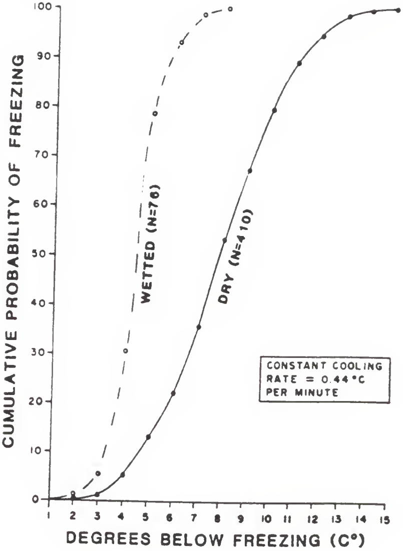

Figure 4-1. Probability of freezing (based on experimental supercooling point tests) of freshly collected monarch butterflies in Mexico (dry) compared with monarchs wetted with over 1 mg of water on the body surface.

Canopy Cover and Rainfall

The 21 less exposed quadrats (as calculated by Apple graphics) had a mean total rainfall of 31 ± 2.1 (SE) ml per cup, significantly less than the 22 more exposed quadrats, with a mean of 41 ± 2.7 (SE) ml per cup (one way ANOVA, $P < 0.005$). These results verify that the canopy functions as a partial umbrella for the monarch clusters beneath, and therefore that overhead canopy increases survivorship during and after winter storms when rain or snow is followed by sub-zero temperatures.

Exposure and Radiational Cooling

Monarchs under the most dense cover (5% exposure) had body temperatures approximately the same as ambient air temperature (measured at the same height), while those more exposed had body temperatures below ambient, in direct proportion to the degree of exposure (Figure 4-2). Multiple regression analysis of these data gives a high coefficient of correlation ($r = 0.98$) with body temperature as the dependent variable and ambient air temperature and percent exposure as independent variables. The predictive equation for body temperature (Tb) given ambient air temperature (Ta) and percent exposure (%Ex) is: $Tb = 1.083(Ta) - 0.034(\%Ex) - 0.98 + E$. Temperatures of completely exposed butterflies were usually 3 - 4 degrees C below ambient air or monarchs under dense cover. Thus, the degree of canopy cover not only affects air temperature beneath the forest (Calvert and Brower, 1981; Calvert et al., 1983), but also affects the temperature of individual monarchs bodies, per se.

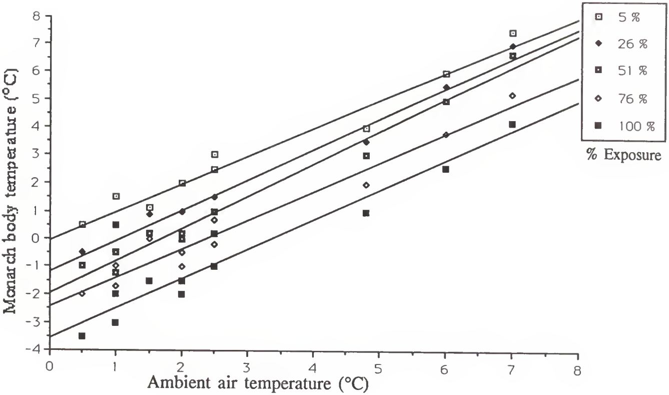

Figure 4-2. Ambient air temperatures vs. body temperatures of monarch butterflies at 04:30 AM (measured over several nights) positioned under covers providing different exposures to the night sky.

Discussion

My present study of the effects of environmental factors on monarch cold-hardiness clarifies and confirms many previous observations. For instance, it was previously found that air temperatures at ground level in the open areas adjacent to a monarch colony were an average of 3.6°C colder than beneath the oyamel firs within the colony (Calvert and Brower, 1981) and 4.4°C colder than at one meter elevation in the open (Calvert and Cohen, 1983). These lower temperatures in open areas and nearer the ground result from radiational cooling of the exposed substrate. Calvert (1982) found that ground temperature declined with distance from a large fir tree — at 12 meters from the tree in a clearing, the ground temperature was 5°C lower than under the tree. This study also demonstrated that forest thinning, a practice of increasing frequency in the area, leads to decreased nightly air temperatures at ground level. Such microclimatic temperature differences can be complex, but are well understood (Geiger, 1980). Monarch mortality due to cold temperatures was reported in four previous studies to be greatly increased (and in most cases associated with increased weight because of dew/frost) when butterflies were left on the ground all night in open areas as compared with those (a) left grounded beneath understory vegetation (Calvert et al., 1986; Calvert et al., 1982), or (b) within the monarch colony (Calvert and Brower, 1981), or (c) perched on stakes a meter off the ground (Calvert and Cohen, 1983). Air temperatures at ground level where much mortality occurred were not drastically low: only -1.7°C on average in one study and -4.9°C in another. Calvert et al. (1986) found that fifty percent of monarchs exposed all night on the ground in open areas suffered loss of flying ability when ambient air temperatures dropped to -3°C. However, Urquhart (1960) reported that he subjected groups of monarchs to 12 hours of sub-zero temperatures, and found complete recovery in some exposed to temperatures as low as -6.8°C .

Based on my measurements of the effects of wetting and exposure on monarch supercooling points, it is clear that the cold mortality previously reported cannot be explained simply by exposure to lower temperatures. As was predicted by Calvert and Brower (1981), innoculative freezing from dew or frost is indeed a major factor in the lethality of cold. The moderate supercooling ability of monarchs, adaptively more effective at night, is significantly reduced if surface moisture is present. The effect of radiational cooling is also considerable, especially if monarchs are also wet, as tends to occur to exposed butterflies during the winter storms or from dew. The experimental SCP tests indicated a body temperature of -4.18°C at which half of wetted monarchs would probably be frozen (LT-50). If body temperatures of exposed butterflies are 3-4 degrees below ambient, then the critical ambient LT-50 for wetted, exposed butterflies is around 0°C. The probable interaction of the effects of wetting and exposure is shown in Figure 4-3. Shelter from wetting and the cold night sky seems to be very important in the ability of monarchs to survive at the Mexican overwintering sites, and quite possibly during the migration as well.

Selection forces resulting from this interplay of cold temperatures and ecological factors would seem strongly to favor the evolution of shelter-seeking behaviors at the overwintering sites. Along with predator pressure (Brower and Calvert, 1985b) and facilitation of successful mating (Van Hook, In Press), these selective forces may have contributed to the clustering behavior and the choice of the oyamel ecosystem as overwintering refuge. Rational strategies for the conservation of the migratory phenomenon must take into consideration these ecological factors in relation to the physical cold-hardiness of monarchs. An intact forest canopy that affords shelter from the night sky and some protection from wetting is clearly critical to the survival of overwintering monarch butterflies.

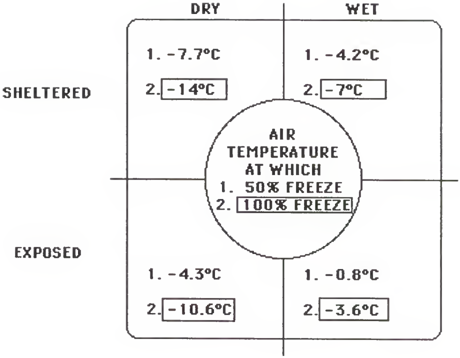

Figure 4-3. Both wetting and exposure affect the cold-mortality of monarch butterflies in Mexico, as determined by experimental manipulation and SCP testing. These environmental factors occur simultaneously in many cases, and probably interact as depicted above. Thus, the air temperature at which there is 50% mortality under dry, sheltered conditions is -7.7°C, but when the butterflies are wet and exposed, 50% mortality is expected at -0.8°C.

CHAPTER 5
BIOCHEMICAL CORRELATES OF FREEZE-RESISTANCE IN OVERWINTERING MONARCH BUTTERFLIES IN MEXICO

Introduction

The fact that water freezes poses a harsh problem for terrestrial poikilotherms. Intracellular ice formation is usually lethal to cells (Somme, 1982). Some species of poikilotherms avoid sub-zero temperatures by migration or retreat to appropriate hibernacula. Others have evolved ingenious biochemical methods of preventing or surviving the formation of ice inside their bodies.

In the case of the monarch butterfly, Danaus plexippus L., standard cryobiological tests showed both a seasonal change in SCP and a daily change at the overwintering site in Mexico. I carried out chemical analyses of monarchs from (1) different parts of their migratory cycle and (2) from different times of day at the Mexican overwintering sites to attempt a biochemical explanation for both these empirically observed phenomona. Since the most commonly reported cryoprotectant is glycerol, I began with assays for this polyol. Subsequently, I analyzed samples of monarchs for total sugars and component carbohydrates.

Methods

Samples

Monarch butterflies from different parts of their migratory cycle were analyzed for glycerol and sugars at the University of Florida. An initial sample (n = 37) analyzed for glycerol was sent alive on ice from Mexico in February, 1984. All the other samples from Mexico were collected January 14 through February 27, 1986, from the overwintering site on the Sierra Chincua near Tuxpan, Michoacan. These butterflies were kept no longer than a day outside in nylon mesh cages before being tested for supercooling point (SCP), immediately after which they were weighed, sealed in labled cryo-vials and dropped into a large flask of liquid nitrogen. In Florida, the samples were kept frozen until analysis. The sample from Cape San Blas, Florida (n = 12) was collected 16 November 1984, and transported on ice to the University of Florida for glycerol analysis. The summer sample from Gainesville, Florida (n = 20) was raised in the laboratory at approximately 22°C and ambient photoperiod. Some of these were tested for SCP after eclosion, and the rest were tested 3 days after eclosion. All were kept under ambient lab conditions until testing for SCP, and then were weighed and kept frozen until analysis by TLC. A summary of sample information and methodology is shown in Table 5-1.

Glycerol Analysis

Glycerol was measured using an enzyme method modified from Hagen and Hagen (1962). Each butterfly (having been previously weighed) was dried for 16 hours in a forced draft drying oven at 60°C, weighed again and homogenized in 10 ml chloroform. The water soluble components were extracted twice with 2.0 ml distilled water. After each extraction the mixture was centrifuged at 5000 RPM for 5 minutes, and the aqueous layer was removed and pooled. The volume of this pooled extract was then adjusted to 5 ml. A pair of 10 ml tubes were prepared for each sample, and into each tube was added 0.5 ml of

Table 5-1. Samples of monarch butterflies analyzed for biochemical correlates of freeze-resistance.

Assay	Sample	Time	Method	n
Glycerol	Florida	1984	Enzyme	12
	Mexico	1984	Enzyme	37
	Mexico	1986	Enzyme	33
	Mexico	1986	TLC	36
	Florida	1986	TLC	20
Total Sugar	Mexico	1986	Anthrone	65
Component Carbohydrates	Mexico	1986	TLC	36
	Florida	1986	TLC	20

the water extract and 1.3 ml of a NAD-glycine buffer. This buffer was prepared fresh before each assay by dissolving NAD (from Fisher Scientific) in 0.1 M glycine buffer (pH 9.5) at a concentration of 6.65 mg/ml. To one of the tubes of each pair (the control) was added 0.2 ml water, and all tubes were put into a water bath at 30°C. At time zero for each pair, 0.2 ml of enzyme (glycerol dehydrogenase, from Calbiochem., diluted to a conc. of 10 units/ml) was added to the other tube of each pair (the experimental). After 30 minutes in the water bath, the contents of the tubes were transferred into 2 ml cuvettes and read at 340 nm in a Perkin Elmer spectrophotometer. Time was recorded for each sample at addition of the enzyme, and optical densities were recorded after exactly 30 minutes. The difference in O.D. between the experimental and control is directly equivalent to the concentration of glycerol in each sample, since the action of glycerol dehydrogenase on the glycerol present reduces NAD to NADH+ proportionally, which causes an increase in O.D. at 340 nm. Standards of 0.01, 0.05, 0.1, 0.5, and 1.0 mg/ml glycerol were prepared with each assay and treated exactly the same as samples. Concentrations of glycerol in each sample were calculated by comparison with the standard curve (regression of Δ O.D. to standard concentrations). Each sample concentration (mg/ml of the extract) was multiplied by 5 (total ml of extract) for total mg glycerol in each monarch. This total glycerol was divided by the weight of water (initial wt. - dry wt.) in that butterfly, and expressed as mg per10 ml of body fluids.

Analysis of Sugars

Separation of lipids, sugars, and polyols in preparation for carbohydrate analysis and thin-layer-chromotography followed the methods of Ring and Tesar (1980) and Van Handel (1965). Each butterfly sample was weighed, freeze-dried and reweighed to obtain total water content. Dried butterflies were homogenized in 10 ml of a mixture of saturated sodium sulphate, chloroform, and methanol (0.8:1:2) in a thick walled glass tube submerged in a melting ice bath. After centrifugation at 5000 RPM for 20 minutes, the supernatant was decanted into a large test tube (A) and the residue extracted again with 5 ml of water, chloroform, and methanol (0.8:1:2). After another centrifugation, both supernatants were pooled in tube A. Two ml of a water-chloroform mixture (1:1) was added, the contents were throughly mixed, and a short spin at 5000 g aided the formation of a two-phase system. The top aqueous layer, containing sugars and amino acids, was transferred with a pasteur pipette into test tube B. The remaining layer of nonpolar solvent in tube A was saved for lipid analysis.

Total amounts of sugars and glycogen were determined by the anthrone reagent assay (Ring and Tesar, 1980). Anthrone reagent was prepared by pouring 760 ml of concentrated sulfuric acid (d=1.84) while stirring and cooling, into 300 ml water. Immediately before each assay, 150 mg of anthrone was mixed with 100 ml of the sulfuric acid solution. For the assay, 0.1 ml aliquots of the aqueous solutions in tubes B (after first determining the volume in each tube) were mixed with 3 ml of the anthrone reagent (Van Handel, 1965) and heated in a shaking water bath at 90°C for 20 min. Aliquots of glucose standards of known concentrations were added to anthrone reagent and heated along with the other samples. All tubes were then cooled in chilled water and optical density was read at 620 nm.

For thin layer chromotography of sugars and polyols, the extracts in tubes B (from above) were evaporated to dryness in a 60°C water bath under a stream of nitrogen, and reconstituted in 1 ml distilled water. Known amounts of these samples (10 or 15 µl) were spotted on 7G silica gel plates, along with 2.0, 1.5, 1.0, 0.5 and 0.25 µg of glucose, fructose, sucrose, trehalose and glycerol. The plates were developed in a chloroform-methanol-water (64:36:8) solvent system, dried, and sprayed (under a hood) with a thymol-H_2SO_4 reagent. This reagent was prepared fresh each time by adding 5 ml conc. H_2SO_4 to a solution of 0.5 g thymol in 95 ml ethanol (Kartnig and Wegschaider, 1971). The plates were then heated at 120°C in a drying oven for 10 min for sugars and 20 min for glycerol, and allowed to cool for 5 min. After measuring for Rf values, all spots were scraped off into 10 ml tubes, and the color was eluted with 4 ml concentrated formic acid. The tubes were centrifuged to remove the silica gel, decanted into cuvettes, and read on the spectrophotometer at 510 nm for sugars and 340 nm for glycerol.

Results

Glycerol

The results of analyses for glycerol (both by enzyme method and TLC) of monarchs from different parts of the migratory cycle are summarized in Figure 5-1. The concentration of glycerol in monarch body fluids was found to be significantly lower in Florida monarchs collected in the summer and autumn than in overwintering monarchs in Mexico. This pattern reflects the pattern of SCP change found seasonally in these butterflies.

However, the daily fluctuation in freeze-resistance observed in Mexico cannot be explained by changes in glycerol concentrations. Figure 5-2 shows SCP data from Mexico, 1986, grouped for three daytime intervals at which they were frozen (Morning, 7-10; Noon, 10-13; and Night, 17-22) and glycerol concentrations of monarchs that were tested for SCP at these times of day and kept frozen until analysis. The glycerol concentrations (expressed as mg/10 ml for juxtaposition with SCP) tend to increase slightly through the day, with the mean concentration at night significantly higher than in the morning (ANOVA, Scheffe F test, $P < 0.05$). Regression of individual SCPs against glycerol concentrations of the same monarchs is not significant at the $P \leq 0.05$ level.

Total Sugars — Overwintering Monarchs

Figure 5-3 summarizes the results of the anthrone assay for total sugars on overwintering monarchs that were tested for SCP in Mexico and kept frozen until analysis.

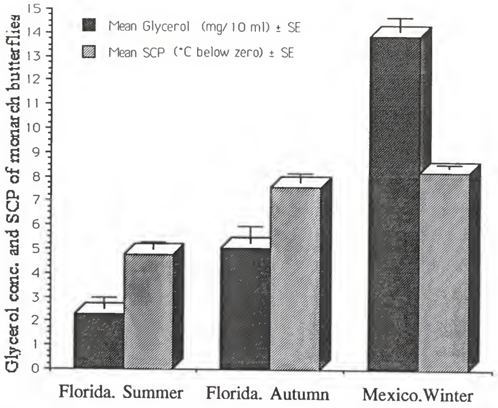

Figure 5-1. Mean glycerol concentrations (mg/10ml) and mean SCP (degrees below zero) of samples of monarch butterflies from different parts of their migratory cycle. Bars = S.E.

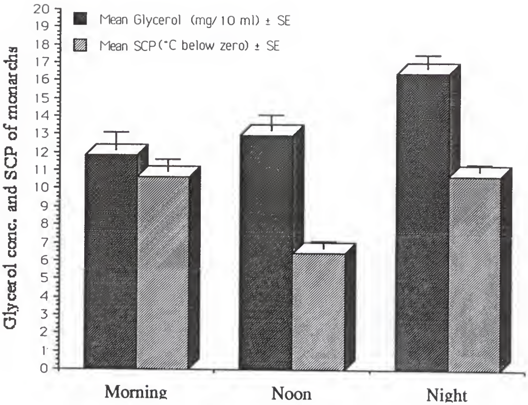

Figure 5-2. Mean glycerol concentrations (mg/10ml) and mean SCP (degrees below zero) of samples of overwintering monarch butterflies in Mexico tested for SCP at different times of day. Bars = S.E.

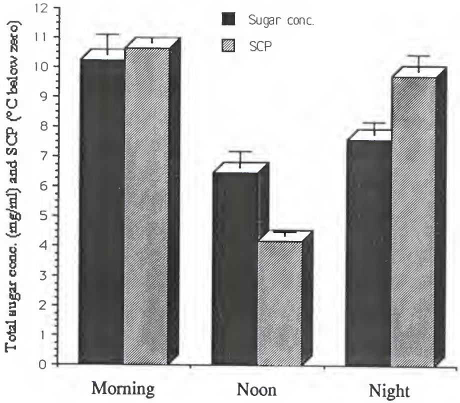

Figure 5-3. Mean total sugar concentrations (mg/ml) and. mean SCP (degrees below zero)of overwintering monarch butterflies. Bars = S.E.

The mean total sugar concentration in the morning is significantly different from the means of the other two groupings (One-way ANOVA, Scheffe F test, $P < 0.05$). Furthermore, regression of total sugar concentrations against the SCPs of these samples was significant ($P = 0.0016$, $y = 0.6x - 3.53$, $r = 0.62$).

Component Carbohydrates

The TLC analysis revealed three major carbohydrates: trehalose, glucose, and glycerol, with Rf values of 0.18, 0.36, and 0.64, respectively. These were identified by comparison with known standards run simultaneously. Figure 5-4 shows the mean concentrations (all expressed as mg/ml of body water) of these compounds for the same time of day groupings on monarchs tested for SCP in Mexico. The glucose concentration tends to fall throughout the day, with the mean concentration of the night group being significantly lower than that of the morning group (ANOVA, Scheffe F test, $P < 0.05$). The glycerol pattern by this analysis shows no significant changes during the day. The means of the daytime groups were compared with one-way ANOVA. However, the trehalose concentrations of the night group were significantly higher than the morning and noon groups (which did not differ statistically). Regression analysis of concentrations of each of the three component carbohydrates vs SCPs of the respective monarchs showed no significant relationship in the case of glucose and glycerol; however, a significant correlation was found ($P = 0.014$) for trehalose concentration vs SCP ($n = 36$, $y = -0.45x - 7.55$, $r = 0.41$).

Figure 5-5 shows the relationship between levels of trehalose, glucose, and glycerol of 20 monarchs raised in the summer in Gainesville, Florida, compared with levels of these carbohydrates in overwintering monarchs in Mexico (n=36). The concentrations (expressed as mg/100 ml) of trehalose and glycerol are significantly higher in the Mexican sample than in the Florida sample (One way ANOVA, Scheffe F test, $P < 0.05$). The glucose levels are not significantly different.

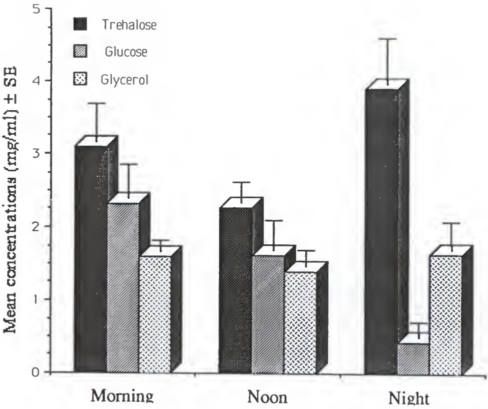

Figure 5-4. Mean concentrations (mg/ml) of primary carbohydrates of overwintering monarch butterflies at different times of day. Bars = S.E.

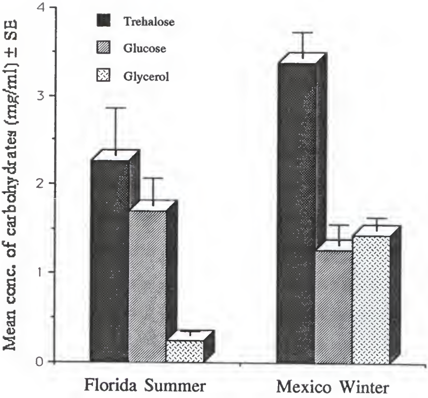

Figure 5-5. Mean concentrations (mg/ml) of primary carbohydrates in monarch butterflies at two points on their migratory cycle: Florida, July, 1987, and Mexico, Feb, 1986. Bars = S.E.

Discussion

Seasonal Cold-hardiness

Gycerol concentrations were higher by almost six-fold in overwintering monarchs in Mexico compared with summer monarchs in Florida. This difference is associated with a moderate increase in supercooling capacity. In studies of insect cold-hardiness, this type of correlation is considered explanatory, and certainly a great many investigators have found glycerol associated with seasonal freeze-resistance in insects. However, it would be incautious to accept this association as more than a partial explanation, especially since glycerol concentrations in Mexico do not correlate significantly with SCPs of individual monarchs. Also, the levels of glycerol, while definitely increased in autumn and in winter, are still low compared with many other reported values for cold-hardy insects. Some eggs and pupae have been found to accumulate as much as 20% of fresh body weight as glycerol (Ring and Tesar, 1981). On the other hand, some reports link cold-hardiness with increased glycerol levels that are equivalent to those seen in monarchs. For example, exposure of Galleria mellonella pupae to cold temperatures will induce an increase in hemolymph glycerol concentration from 64 to 135 mg/100 ml (Marek, 1979).

Levels of trehalose are also significantly higher in overwintering monarchs than in monarchs from the Florida summer. In this case, both the total sugar concentrations and trehalose levels show significant correlations with SCPs of individual monarchs in Mexico (none of the carbohydrates is correlated with summer SCPs, however). Recently, several authors have reported multifactorial cryoprotective systems, which may be as effective and less toxic than buildup of any one component (Ring, 1980). In larvae of Scolytus ratzeburgi, glycerol, sorbitol, glucose, and trehalose all were found to contribute to cold-hardiness (Ring, 1977), and glycerol, mannitol, and trehalose concentrations were related to the freeze protection of Crytopygus antarcticus (Somme and Block, 1982). In the present study, the increased levels of glycerol and trehalose probably both contribute to the cold hardiness of overwintering monarch butterflies in Mexico.

Circadian change in SCP — possible mechanism.

As well as a seasonal increase in cold-hardiness and glycerol content, monarchs in Mexico exhibited a daily SCP fluctuation correlated with levels of total sugar and trehalose. One possible source of glycerol, glucose, and trehalose in insects is stored glycogen in the fat body, a dispersed internal organ responsible for storage and metabolism of lipids, carbohydrates, and proteins (Kilby, 1963; Zeigler et al., 1979). Although biosynthesis of small molecular weight alcohols (polyols) can procede from several substrates, it is generally accepted that a major source of the polyols in freeze-resistant insects is glycogen (Hayakawa and Chino, 1982). For example, in diapausing pupae of the cecropia silkmoth, Hyalophora cecropia, exposure to cold stimulates conversion of glycogen to glycerol (Zeigler and Wyatt, 1975). A few insects such as the saw fly (Asahina and Tanno, 1964) and diapausing silkworm pupae (Hayakawa and Chino, 1981), accumulate large amounts of trehalose (with little polyol increase) in the hemolymph during winter (also see Morrissey and Baust, 1976; Ring, 1977; Shimaka et al., 1984; Somme and Block, 1982; Tanno, 1964). In the silkworm, Philosamia cynthia, most of the glycogen initially present in the fat body is converted to trehalose on exposure to low temperatures of 2°C, and the reverse reaction, trehalose to glycogen, occurs when the pupae are subjected to higher temperatures of 20-25 °C (Hayakawa and Chino, 1981).

Two enzymes considered to function as key enzymes in the breakdown and resynthesis of glycogen are glycogen phosphorylase and glycogen synthase (Fischer et al., 1971; Murphy and Wyatt, 1965). In the above mentioned study of silkworm pupae (Hayakawa and Chino, 1982) these two enzymes were found to be important in the (multistep) interconversion of glycogen and trehalose. Both enzymes exist in an active form 'a' and an inactive form 'b'. At warm temperatures, glycogen synthase b (inactive) is transformed to synthase a (active) while glycogen phosphorylase 'a' (active) is converted to phosphorylase 'b' (inactive). These shifts in enzyme activity accompany conversion of trehalose to glycogen when the pupae are at higher temperatures. Cold temperatures stimulate the conversion of glycogen to trehalose by causing phosphorylase 'b' (inactive) to shift into the active form 'a' and the change of synthase 'a' (active) to synthase 'b' (inactive). The authors showed that this conversion of enzyme activity happened within half a day of the change in ambient temperature. Interestingly, the transformation of glycogen phosphorylase 'b' into the 'a' form by cold was first demonstrated (Zeigler and Wyatt, 1975) for fat body from diapausing pupae of H. cecropia, in which the final result of glycogen breakdown was glycerol.

The pattern of SCP change observed in the overwintering monarchs could very well be the result of a similar mechanism, if the enzyme conversions occurred quickly enough. It could also be true that the switching of active to inactive forms of these enzymes is part of an ongoing circadian mechanism that functions to track the day-night periodicity rather than simply respond to the temperature changes. Glucose and trehalose (along with uric acid and serotonin) are the only components of insect plasma for which a circadian rhythm in concentration has been demonstrated, but the peak concentration of trehalose in Periplaneta, the test animal, occured 2-3 hours before daylight (Woodring, 1985).

The results clearly implicate glycerol and trehalose as cryoprotectants in overwintering monarch butterflies. The pattern of fluctuation in trehalose concentration is correlated with both seasonal and daily SCP changes. Furthermore, the temperature dependent interconversion of fat body glycogen to trehalose that operates in silkworm pupae is a mechanism that could easily explain these daily changes, either in response to temperature changes or as a circadian mechanism that anticipates nightly cold.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Summary of Findings

The monarch butterfly is the only member of the subtropical family Danaine to range over most of temperate North America. Virtually all the monarchs in the U.S. and Canada migrate each winter to sites in Mexico and California (with a small population on the west coast of Florida), as one defense against cold weather. Migration, however, does not give complete freedom from cold, because the monarchs that fly to Mexico come together in overwintering colonies on mountain sides at high altitude. Occasional, severe winter storms occur in these areas, but surprisingly there has been relatively low butterfly mortality (Calvert et al., 1983). This and similar observations stimulated the present study of monarch cold-hardiness.

Of all known insect cold-hardiness agents, thermal hysteresis proteins (THPs), since they would block innoculative freezing, would seem to be most effective under the climatic conditions at the overwintering sites. However, I found no convincing evidence for such a mechanism. Comparative data from standard cryobiological tests performed in Florida and Mexico showed monarchs to be freezing-susceptible with a moderate seasonal increase in supercooling ability. During the second field season on the Sierra Chinqua in Mexico, I discovered that the overwintering monarchs had lower supercooling points (SCP) early in the morning and at night than in the day, a presumably adaptive pattern previously reported for an Andean plant, but never before in insects. The fact that treatment with methoprene, an insect juvenile hormone analogue, altered the daily pattern of SCP change is evidence that links the cold-hardiness with hormonal factors already known to orchestrate the monarch migration/diapause syndrome.

Two ecological factors at the Mexican overwintering sites were found to be very important in the cold-survival of monarchs. Water on the body surface caused monarchs to freeze at higher sub-zero temperatures than other butterflies not wetted. Also, radiational cooling to the night sky caused the body temperatures of exposed monarchs to drop approximately 4°C below ambient air temperature. Both these factors logically interact when exposed butterflies are wetted with dew or rain, resulting in much higher risk of freezing under such conditions. An intact forest canopy acts to moderate exposure and wetting, and therefore is very important in protecting the clustering monarchs from nightly cold and the occasional winter storms that impact the areas.

Biochemical analyses of monarchs captured during different parts of the yearly cycle showed that concentrations of two carbohydrates, glycerol and trehalose, fluctuated seasonally with changes in supercooling ability. However, only trehalose concentrations correlated significantly with the individual SCPs of overwintering monarchs tested at different times of day. Glycerol has been commonly found as a cryo-protectant in insects, and trehalose has been reported in this role in several instances. Probably both of these substances add to the net supercooling ability of overwintering monarchs, with daily shifts in trehalose concentration accounting for the circadian change in SCP observed in Mexico.

Directions for Future Research

Some major questions have been answered by this study. However, several others have been raised, and can now be addressed more clearly. First, some details remain to be added for confirmation and higher resolution of the general hypotheses supported by these findings. Secondly, some new hypotheses and speculations have been suggested.

Questions of Resolution

(1) The initial prediction that THPs might be present in a monarch cold-hardiness physiology (in the best of all evolutionary scenerios) was logically discarded for lack of convincing evidence, but the slight but measurable thermal hysteresis (less than 0.1°C) remains unexplained. I think that this result was a byproduct of the experimental procedure I followed, which defined freezing point in terms of observed crystal growth and melting point in terms of observed crystal shrinkage. It was slightly easier to discern growth from shrinkage, which probably led to the measured hysteresis. Another contributing factor is that only 0.2°C/min cooling/warming rate could be achieved with the cooling bath, not the 0.1°C/min recommended in the literature. These experiments should be repeated with more precision if such a slight thermal hysteresis is to be believed.

(2) Another aspect of the thermal hysteresis question should be checked. These experiments were done the first field season in Mexico, before a circadian change in freeze-resistance was detected. All tests for THPs were done in the daytime. It is remotely possible that nightly determinations might show more hysteresis. However, some of the experiments showing the effect of wetting on SCP were done at night, and just as in the day, water on the body surface caused innoculative freezing at higher temperatures (in both cases, wetted SCPs were -3 to -4°C). If THP concentration increased at night, the nightly wetting experiments would have given different results.

(3) I continually attempted in this study to carry out the comparative cryo-assays as soon as possible with animals freshly collected from natural conditions, and to keep samples frozen until just before chemical analysis. By this method, any possible lability of physiological state could be minimized. Another approach would be to subject a lab colony of monarchs to a controlled environment, changing day length and temperature in order to artificially provoke diapause and cold-hardiness. This would require a sophisticated (and large) environmental chamber, but it would allow the daily SCP change to be investigated

in more depth, and the question of an endogenous vs. entrained basis for the circadian change could be answered.

(4) Another question that could be answered with a slightly higher level of technology is the relationship between rate of cooling and SCP. The Haake bath could only implement one rate of cooling. Since this rate was constant throughout the study, comparative differences are valid. However, the connection between lethal limits shown by my experiments and those of the real world will remain an approximation until the effect of cooling rate is quantified. Baust and Rojas (1985) levy this criticism at the whole of the cryobiological literature, and as far as I know, no one has looked carefully at the problem. Similarly, the duration or time the animal is exposed to a temperature below the freezing point of body fluids should affect the SCP, but again, no methodical assessment of this variable can be found in the literature.

New Hypotheses

(1) The effect of methoprene on the daily SCP pattern in Mexico was puzzling, and indicates a complexity that would be extremely interesting to tease apart. The SCPs of treated monarchs were elevated (compared with controls) in the afternoon and night, as was predicted if high JH levels mediate a summer reproductive state (and summer SCP). But in the morning, there was no effect of treatment. An explanation may lie in the dynamics of hormonal changes going on through the day. A simple hypothesis is that JH increases during the day and is degraded by JH esterase in the afternoon. In this case, treatment with methoprene in the morning might be surplus, able to change the system no faster than it is already being shifted. In the afternoon, methoprene might not be sensitive to JH degradation, and possibly acts quickly on a system experiencing waning JH levels. This scenerio may not be true, but in any case a close examination of the hormonal mechanisms in control of the situation would be another large project, and a very interesting one. One diagnostic experiment that could be tried is treatment with precocene, a chemical that

destroys the corpora allata. This should produce very low JH levels and, if this controls cold-hardiness, very low SCPs as well. Assays for JH and JH esterase on monarchs from different times of day should reveal whether JH levels are fluctuating on a daily cycle.

(2) The mechanism behind the daily changes in trehalose concentration could be similar to that reported for silkworm pupae, where the enzymes controlling glycogen/trehalose conversion in the fat body undergo temperature dependent shifts in activity (Hayakawa and Chino, 1981; Hayakawa and Chino, 1982). Enzyme assays on overwintering monarchs sampled from different times of day under both ambient and cue-deprived conditions would likely resolve this hypothesis.

(3) Rankin (1978, 1978) and Dingle (1978, 1986), among others, have investigated hormonal modulation of the life cycle in the Milkweed bug, Oncopeltus fasciatus, an animal ecologically similar in many ways to the monarch butterfly. Herman (1981, 1985) has also worked on monarch hormones, so that the stage is set for a comparative study. If populations of each of these animals could be subjected to controlled environmental conditions and then tested for cold-hardiness and simultaneously assayed for hormonal state and various biochemical parameters, the results might be useful in the ongoing attempt to understand the evolution of insect life cycles (e.g., Dingle, 1986; Tauber et al., 1986).

(4) SCPs and concentrations of carbohydrates in this study showed considerable variation. In drawing inferences, this variation was subjected to various statistical tests, and, depending on the outcome, treated as either an indication of insignificant relationship or as noise to be squelched in order to see a validated pattern. However, heritable variation is the prerequisite for evolution, and if some of the variation in SCP/cryoprotectant values is genetically based, this means that the possibility for evolving increased cold-hardiness lies in the monarch genome. Design of controlled experiments for this question, however, soon becomes problematic for two reasons. By present methods, determination of cold-hardiness and/or levels of cryo-protectants require sacrifice of the butterfly. Also, cold-hardiness is an attribute of winter diapause, whereas reproduction involves another

physiological state. It might be possible to (1) first raise monarchs under reproductive conditions and allow mating, (2) collect and keep track of offspring, (3) then subject the parental monarchs to diapause inducing conditions and (4) test for cold-hardiness and cryoprotectants. On the other hand, an assay for trehalose/glycerol, or total sugar concentration of small hemolymph samples might be used as an indicator of cold-hardiness for living monarchs. In this case, the butterflies with the highest sugar content during winter conditions could be chosen for breeding stock for a few successive generations, with samples of progeny tested for SCP.

However, a large scale, uncontrolled experiment may be in progress. If the efforts are not successful in preventing further anthropogenic degradation of the Mexican overwintering sites, the fir forests will be thinned and reduced (Snook, In Press). My evidence shows that this will probably result in increased cold hazard for the monarchs (also see Calvert et al., In Press) and increased selection for cold-hardiness. The ultimate result depends on the speed and severity of changes, on the heritability of cold-hardiness, and on exactly how the different component systems of physiology and behavior in the functional life-history pattern respond to the selection pressure.

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BIOGRAPHICAL SKETCH

James Anderson grew up in rural Mississippi, beginning school at five in a one-room schoolhouse and experiencing pre-mechanized agriculture at close range. In this bucolic environment he came to detest whining country music and proselytizing Baptists, but developed a great fondness for the natural world. After graduating from Mississippi State University in 1967 at age twenty, he attended medical school at the University of Mississippi just long enough to prove to himself that he could make the grade. He then sought a more eclectic education. First it was necessary to serve two years as a Lieutenant in the U.S. Army. After this he spent a few wanderyears, drifting through several occupations and landscapes. Finally he returned in 1977 to the study of biology at East Tennessee State University, earning an M.S. in 1979. Before coming to the University of Florida, he worked as a research associate at the Quillan-Dishner College of Medicine. Along this winding path, he married Paula Morgan and engendered in 1983 a daughter, Morgan Anderson.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the Department of Zoology in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1990

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